DOCTOR OF SCIENCES

Design of an immunization strategy based on recombinant proteins of the measles virus in the context of a changing epidemiology

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Design of an immunization strategy based on recombinant proteins of the measles virus in the context of a changing epidemiology

Thèse présentée par

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“Our doubts are traitors, and make us lose the good we oft might win, by fearing to attempt.”
William Shakespeare, Measure for measure.

Je souhaite dédier le présent manuscrit à la mémoire de mon père

Daniel DAMIEN
20/02/1950 – 5/03/2002

Pendant plus de 35 ans, mon père a travaillé sans relâche pour mettre sa famille à l'abri du besoin et assurer la meilleure éducation pour ses enfants.

Il nous a inculqué l'amour du travail bien fait, la gentillesse et le respect des autres.
Il avait toujours l'esprit ouvert, il aimait découvrir et voyager.

Son grand cœur fut à la fois sa plus grande qualité et la faiblesse qui l'a emporté.

Il fut, et restera pour toujours, un exemple pour mes frères et moi.

Le travail que vous tenez entre les mains est donc un peu le sien.

Je regretterai toute ma vie n'avoir pas été capable de terminer ma thèse avant que la maladie ne le rattrape, mais j'espère malgré tout qu'elle témoignera de l'accomplissement de sa mission.

Au revoir, voyageur…

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List of abbreviations:

APC:	Antigen-presentating cell
APS:	Ammonium persulfate
Arg:	Arginine (R)
Asp:	Aspartic acid (D)
ATCC:	American type culture collection
BCE:	B cell epitope
BSA:	Bovine serum albumine
CIP:	Calf intestine phosphatase
CD46:	Cluster of differentiation 46
CDC:	Center of disease control
CDV:	Canine distemper virus
CTL:	Cytotoxic lymphocyte (CD8+ T Lymphocyte)
DMEM:	Dubbleco's Modified Eagle Medium
DNA:	Desoxyribonucleic acid
DTT:	Dithiotreitol
ELISA:	Enzyme Linked Immunosorbant Assay
EM:	Electron microscopy
EMBL:	European molecular biology lab
EPI:	Expanded Program of Immunization
FACS:	Fluorescence Activated Cell Sorting
FCS:	Fetal calf serum
FITC:	Fluorescein isothiocyanate
Fmoc:	9-fluorenylmethoxycarbonyl
Gln:	Glutamine (Q)
Glu:	Glutamic acid (E)
Gly:	Glycine (G)
HI:	Hemmaglutination inhibition assay
His:	Histidine (H)
HNE:	Hemagglutinin noose epitope
HPLC:	High performance liquid chromatography
IFN:	Interferon
IgG or IgM:	Immunoglobulin G or M
IL-2:	Interleukin 2
IPTG:	Isopropyl-D-thiogalactopyranoside
K:	Lysine (Lys)
LB :	Luria-Bertani broth
Leu :	Leucine (L)
LNS:	Laboratoire national de santé
Lys:	Lysine (K)
M:	Mimotope
mabs:	Monoclonal antibodies
MCP:	Membrane Cofactor Protein (or CD46)
Met:	Methionine (M)
MHC:	Major histocompatibility complex
mRNA:	Messenger RNA
MV:	Measles Virus
NE:	Neutralizing epitope
NK:	Natural killer cell
NT:	Neutralisation test
OD405nm:	Optical density at 405 nanometres
ORF:	Open reading frame
PAHO:	Pan American Health Organization
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reactor

PBMC:	Peripheral blood mononuclear cell
Phe:	Phenylalanine (F)
PMSF:	Phenylmethylsulfonyl fluoride
PCR:	Polymerase chain reaction
PVDF:	Polyvinylidenedifloride
RNP:	Ribonucleoproteic complex
RNA:	Ribonucleic acid
RPM:	Rotation per minute
RPMI:	Rosweli Park Memorial lnstitute medium
SD:	Standard deviation
SDS:	Sodium dodecyl sulfate
SDS-PAGE:	SDS PolyAcrylamide Gel Elecrophoresis
SIR:	Secondary immune response
SFV:	Semilki Forest Virus
SLAM:	Signalling Lymphocytic Activation Molecule (or CDw150)
SDS:	Sodium-dodecyl sulfate
SSPE:	Subacute sclerosing panencephalitis
SGLB:	Stop gel loading buffer
TCM:	T cell medium
TAE:	Tris acetate EDTA
TAP:	Transporter associated with antigen processing
TCE:	T cell epitope
TCR:	T cell receptor
TEMED:	N,N,N',N' Tetramethyethylenediamine
TT:	Tetanus Toxoid
TNF:	Tumor necrosis factor
Trp:	Tryptophan (W)
Tyr:	Tyrosine (Y)
WHO:	World Health Organization

Part I

Introduction

I. Introduction

I.1 History of measles

Measles is a relatively recent disease for humans that probably evolved from an animal morbillivirus. MV most closely resembles the rinderpest virus, a pathogen of cattle (Norrby *et al*, 1985; Sheshberadaran *et al*, 1986) and it is postulated that measles virus (MV) evolved in an environment in which cattle and humans lived in close proximity. Rinderpest is likely to have evolved from a "young cow" disease in herds of ungulates large enough to maintain a continuous supply of susceptible and establish a pattern of endemic infection. Measles is thus a disease of civilization because its emergence has coincided with the domestication of cattle and the first settlements, where the densely populated regions in the Middle East and India were needed to maintain the disease (McNeill, 1976).

The first case descriptions of measles were attributed to an Arab physician, Abu Becr, known as Rhazes of Baghdad. During the 9th century of the Christian era, he first distinguished measles from smallpox. Rhazes refers to measles as *hasbah*, "eruption" in Arabic, and considered it as a modification of smallpox (Hirsh, 1883): He considered that "anxiety of mind, sick qualms and heaviness of heart, oppress more in measles than in smallpox" (Rhazes, 1748). It is also known that the Hebrew physician, Al Yehudi, had come across measles infection as early as in the 7th century (Plotkin and Mortimer, 1992)

Repeated epidemics were recorded in European and Far Eastern populations between 1 and 1200 A.D. During this period, european descriptions of the disease are limited, but it appears that measles spread across the Pyrenees into France with the Saracen invasion of the 8th century. Measles is first mentioned as a childhood disease in 1224 (McNeill, 1976). Initially, measles was thought to be a diminutive form of the bubonic plague but the distinction between measles and smallpox became clearer by the beginning of the 17th century when the annual bills of mortality in London in 1629 listed the two diseases separately (Plotkin and Mortimer, 1992).

Many of the basic principles of measles epidemiology and infection were recognized only during the 19th century by Peter Panum (Panum, 1940) but already in 1757, Francis Home was able to transmit the disease to naïve individuals using blood from patients (Plotkin, 1967). In 1954, Enders and Peebles were the first to grow the virus in culture by inoculating human kidney cells with the blood from David Edmonston, a child with measles (Enders *et al*, 1957). Later, the virus was adapted to other human and non-human cell lines (Katz, 1958; Ruckle and Rogers, 1957). These studies led to diagnostic tests for measles as well as to the development of the first vaccine.

I.2 The measles disease

Measles is transmitted primarily by aerosol originating from mucous membranes of the upper respiratory tract or by direct contact with nasal or throat secretions of infected persons. Measles is one of the most readily transmitted communicable diseases and probably the best known and most deadly of all childhood rash/fever illnesses. The incubation period is about 10 days, varying from 8-13 days from exposure to onset of fever and about 14 days until rash appears. Measles is most contagious 1-3 days before the onset of fever and cough and is minimal after the second day of rash.

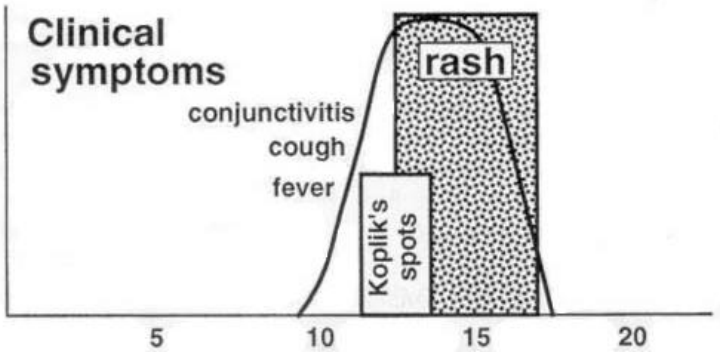

Fig 1: Progression of measles clinical symptoms. Numbers are days after infection

The first signs and symptoms of measles are the warning (prodromal) fever (>38.3°C according to CDC case definition), conjunctivitis (inflammation of the mucous membrane of the eye), coryza (swollen nasal tissue and runny nose), cough and Koplik's spots (tiny red patches with central white specks on the buccal mucosa in which characteristic giant syncytial cells containing viral proteins are identified) (CDC, 1983). After the diffuse secondary spread of the virus, the prodromal symptoms are intensified and the typical red, maculopapular rash appears on the 3rd to 7th day, first on the head and face, and then on the extremities, before becoming generalized, lasting 4 to 7 days after the beginning of prodromal symptoms, and sometimes ending in branny desquamation.

Fig 2: Child with measles rash

The rash is thought to appear as a result of immune T-lymphocytes interacting with infected cells. Children who are T-cell deficient, due to thymic dysplasia, do not develop a rash, but develop extensive giant-cell pneumonia. A reduced white cell count is common (immunodepletion). The disease may be more severe in infants and adults. Moreover, in some cases MV can establish persistent infections of the brain leading to severe neurological complications (Chen *et al*, 1994).

Vitamin A administered to children acutely ill with measles has been shown to reduce mortality and accelerate recovery from pneumonia and diarrhea. Administration of antibiotics is recommended to prevent bacterial complications, and oral rehydration salts for preventing dehydration following diarrhea. Case-fatality rates can be lowered if cases reach health care facilities early enough. For uncomplicated cases, supportive fluids, antipyretics and nutritional therapy may be required. Many children need increased food intake for four to eight weeks to recover their pre-measles nutritional status. Diarrhea and respiratory complications are undoubtedly the most frequent. Uncommon complications include nephritis, myocarditis, pericarditis, hepatitis, ileocolitis and appendicitis.

Encephalomyelitis is the most serious complication, appearing 5-7 days after the rash. This occurs in 1:2000 cases and is more frequent in children over 10 years. The mortality rate is 10%, with permanent mental and physical sequelae reported in 15% to 65% of survivors. With measles encephalomyelitis, the brain shows perivascular hemorrhage and lymphocytic infiltration early in the disease. Areas of demyelination appear later in the brain and spinal cord.

Subacute sclerosing panencephalitis (SSPE) is a progressive, fatal but rare (1 case per 1,000,000 patients) complication of measles. SSPE is an example of a single virus inducing an acute disease and a chronic illness separated by a long interval during which there is restricted synthesis and expression of viral genes. Patients with SSPE display a degeneration of the cortex and the underlying white matter. Perivascular infiltration of plasma cells and lymphocytes, scattered degeneration of nerve cells, hypertrophy of astrocytes, microglial proliferation and demyelination are also evident.

I.3 Epidemiology

Measles occurs worldwide. It has a seasonal appearance and in the temperate climates, outbreaks generally occur in late winter or early spring. In tropical climates, the occurrence increases after the rainy season.

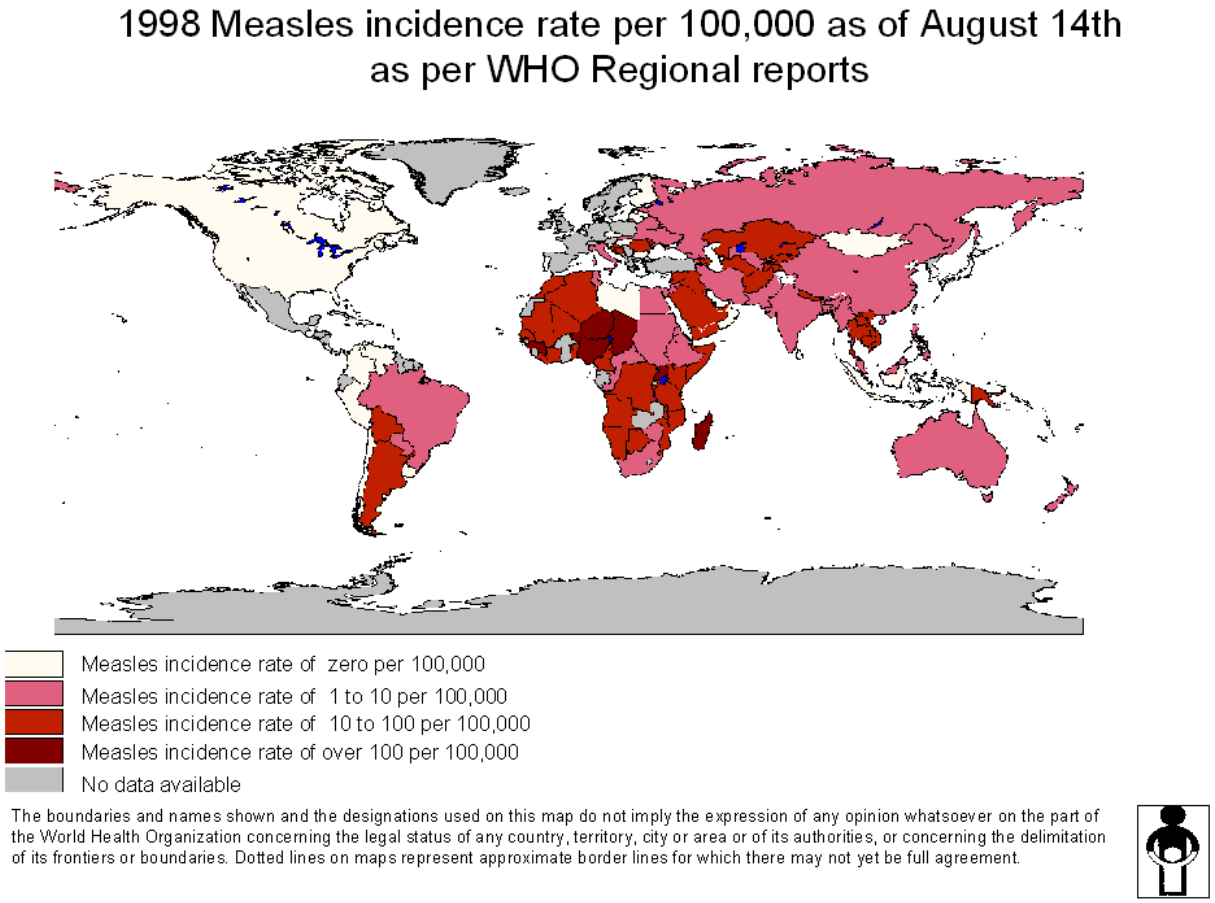

Fig 3: Map of measles incidence in the world (Source: WHO).

Measles is the most contagious disease in humans. With a basic reproduction number of 15, a single patient is able to infect 15 susceptibles individuals. The WHO estimates that over 40 millions cases still

occur worldwide each year, contributing to about 800.000 deaths. In large populations with low vaccine coverage, like many developing countries, measles may be endemic and is occurring within short intervals. The duration is depending on factors such as population size, crowding and immunization status. In countries with high vaccination coverage, measles has a 5 to 7 years periodicity and the case number remains small. As the number of susceptible children becomes large enough to sustain widespread transmission, explosive outbreaks may appear (PAHO, 1999).
Prior to measles vaccination, the measles infection was nearly universal. In 1974, the annual death toll from measles stood at about 8 million and there were an estimated 130 million cases. Immunity to measles was acquired by natural infection or by passive immunization of neonates by maternal antibodies transferred *in utero* to the fetus. This passive immunity wanes gradually as the growing infant dilutes and degrades maternal antibodies. The majority of infants are susceptible to measles by the age of 6-9 months in developing countries and 9-12 months in industrialized countries. After measles infection, the resulting immunity is believed to be protective for life, and measles vaccination has been shown to be protective for at least 20 years (Sutcliffe and Rea, 1996). Since the introduction of measles vaccines in the Americas in the sixties, the number of reported measles cases has declined rapidly. Both industrial and developing countries have experienced more than 99 percent reduction in incidence of reported cases as a result. The creation of the extended program of immunization (EPI) in 1977, and the ensuing increase of vaccination coverage, contributed to a further drop in the number of reported measles cases and a tendency towards longer intervals between epidemics.

However, measles remains endemic in many developing countries, with the largest proportion of cases reported to occur in children under the age of 5 years. In such areas, the interval between the waning of maternal antibodies and the child's exposure to circulating measles virus is short. Measles highest mortality is sustained by children below 1 year of age. Other high-risk groups may include the urban poor; school children (who represent cohorts from previous years when coverage was lower and who may not have been exposed to measles infection); ethnic minorities (who may have been under-served or may have rejected immunization for cultural reasons); hospitalized children (who are at high risk of nosocomial transmission); and children in refugee camps (where crowding facilitates the spread of the virus).

In both developed and developing countries, the epidemiology of measles has changed since the introduction of effective measles vaccines. As vaccine coverage has increased, there has been a marked reduction in measles incidence and an increase of the average age at which infection occurs.

I.4 The measles virus

I.4.1 Description of the virus

The measles virus (MV) is a negative -stranded RNA virus member of the genus *Morbillivirus* in the family *Paramyxoviridae*.

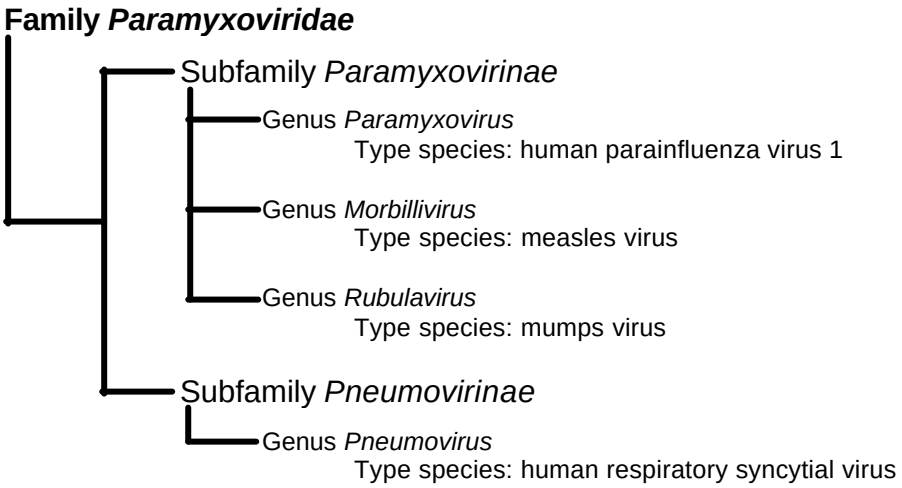

Fig 4: The Paramyxoviridae family.

The morbilllviruses are antigenically stable and they differ from other *paramyxoviridae* by the lack of neuraminidase activity and the formation of intranuclear inclusion bodies. Virions are pleiomorphic (they do not have a defined shape) and their size ranges from 100 nm to 300 nm. The nucleocapsid has a helical structure with a diameter of 21 nm and a central hole of 5 nm (Lund *et al*, 1984).

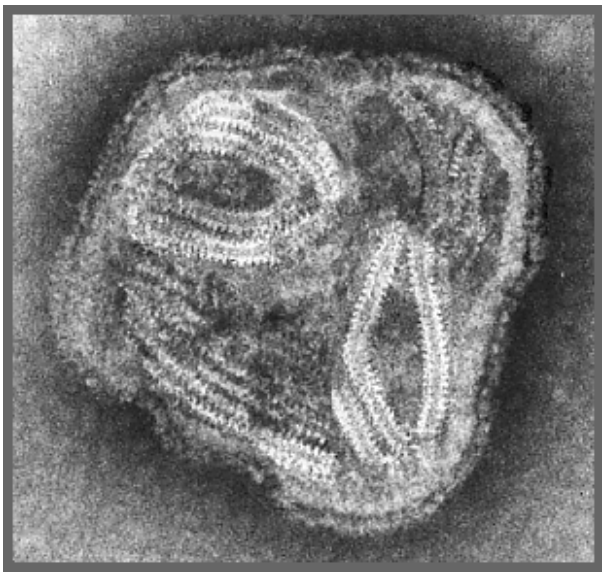

Fig 5: E.M. Picture of measles virus (source: University of Cape Town Website, 1995).

I.4.2 Structure, proteins and cycle of replication

All MV proteins are encoded in the 15894 nucleotides of a non-segmented single-stranded RNA of negative polarity. There are 6 structural proteins: The hemagglutinin protein (H protein, 617 amino-acids), the fusion protein (F protein, 553aa), the nucleoprotein (N protein, 525aa), the phosphoprotein (P protein, 507aa), the matrix protein (M protein, 335aa) and the large protein (L Protein, 2213aa) and two functional proteins only present in the target cell, the C protein (186aa) and the V protein (298aa).

The genome of the measles virus is encoded in a single stranded RNA, surrounded by the N protein, the primary protein of the nucleocapsid. The P protein and the large protein are also associated with the RNA (Lund *et al*, 1984) to form the nucleocapsid, which is packed in the lipid phosphobilayer envelope as a symmetrical coil. On the inside of this envelope, we find the M protein which is though to interact with the nucleocapsid and the two surface proteins H and F.

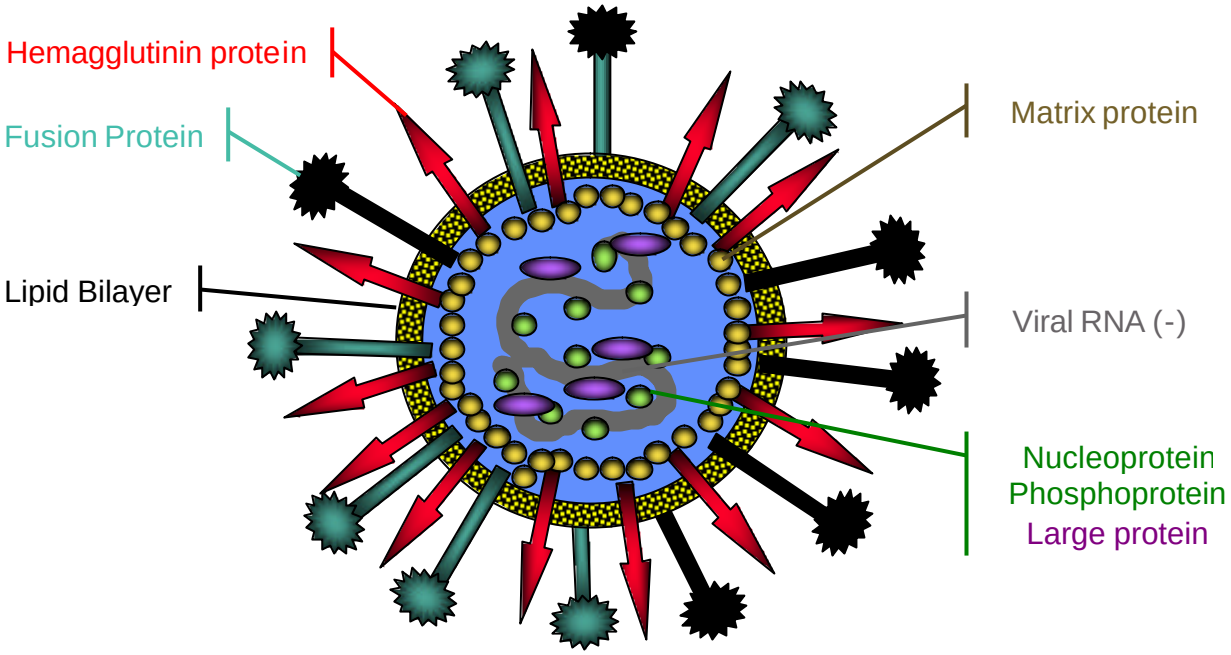

Fig 6: Schematic representation of measles virus.

The MV genome (Alkhatib and Briedis, 1986; Bellini *et al*, 1986; Bellini *et al*, 1985; Blumberg *et al*, 1988; Richardson *et al*, 1986) starts with a 55 nucleotide leader sequence (Billeter *et al*, 1984) that shows a high degree of complementarity to a similar sequence at the 5' end of the genome, suggesting the possibility of the formation of a panhandle sequence (Crowley *et al*, 1988). It also has a non-coding region between the M and F genes. The 6 major structural proteins are encoded by the 6 genes (Fig 7) and the two additional non-structural proteins are coded by differential translation form the P gene.

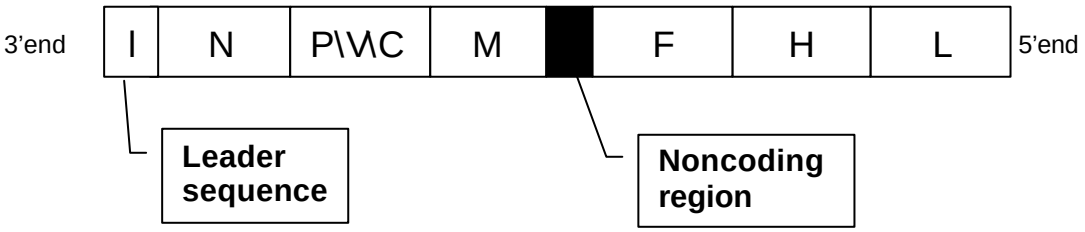

Fig 7: Genome of the measles virus.

I.4.3 Genetic variability

MV is considered as an antigenically stable monotypic virus. Antisera from individuals infected decades ago retain the ability to neutralize current wild-type strains. Nevertheless, MV has an RNA-dependent RNA polymerase with an inherent error rate and no proofreading ability allowing some degree of variability.

Few differences have been described among the current vaccine strains regardless of the geographic origin of the parent virus, suggesting that MV globally was quite similar when most of the parents' strains were isolated (Rota *et al*, 1994). Wild-type viruses are more variable and a number of different lineages of MV are currently circulating worldwide (Ogura *et al*, 1991, Mulders *et al*, 2001).

Measles isolates from many regions of the world have been characterized in parallel studies by our laboratory and by others. In conjunction with classical epidemiologic investigations, these well-characterized viruses have formed a picture of the distribution of wild-type measles viruses in most parts of the world. Eight distinct genotypes have been identified (designated A – H) and the number of genetic groups is likely to increase since the full extent of genetic heterogeneity among wild-type measles viruses is still unknown. Most genetic groups have worldwide distribution except for two of the groups (Clades B and H) which appear to have a more limited circulation (Bellini and Rota, 1998). Clade A consists of vaccine and vaccine-related wild-type viruses (Rota *et al*, 1994), and it is thought to have been a major genotype before routine vaccination was introduced in the late 60s. However, before the introduction of the B95a cell line, which is 10.000 times more efficient for MV isolation (Kobune *et al*, 1990), most MV were isolated on Vero cells, which may have introduced a selection bias.

In most cases, genetic characterization of wild-type measles viruses has been conducted by sequencing the genes coding for the hemagglutinin (H) protein or the nucleoprotein (N). Of the six genes on the viral genome, the H and N genes are the most variable. Over their protein coding regions, the H and N genes vary by up to 7% at the nucleotide level. The single most variable part of the measles genome is the 450 nucleotides that code for the COOH-terminus of the N protein, where nucleotide variability between various wild-type viruses can approach 12%. Several laboratories including our own have analyzed the sequences of wild-type measles viruses and assigned the viruses to various genetic groups (Rota *et al*, 1996; Taylor *et al*, 1991b; Mulders *et al*, 2001).

Despite sequence variability, the genetic groups are still antigenically similar, allowing cross-protection from viruses of all groups using current vaccine strains developed from one of those groups. However cross-neutralization studies indicate that sera from recently infected individuals neutralize some wild-type viruses six to eight times better than they neutralize the vaccine strain, this could be explained by a selective pressure in favor of viruses genetically distant from the vaccines strains (Bellini *et al*, 1994). This phenomenon may lead in the future to the emergence of antigenic variants of the measles virus.

I.4.4 Biological properties of the structural proteins of MV

I.4.4.1 The hemagglutinin protein (H protein)

The H protein is one of the two surface proteins of the MV. It is a type II transmembrane glycoprotein that consists of 617 amino acids. The hydrophobic transmembrane domain of the N terminus acts as a signal sequence for targeting the protein to the endoplasmic reticulum, as well as a membrane anchor

(Hummel and Bellini, 1995). The main function of the H protein is to initiate the infection by binding the virus to the receptors on the target cell (Varsanyi *et al*, 1984), and it cooperates with the F protein to mediate the fusion of the virus with the membrane of the target cell (Schmid *et al*, 1992; Taylor *et al*, 1991a; Wild *et al*, 1991). It is therefore not surprising that the H protein is the main target of neutralizing antibodies and a large number of B-cell epitopes have been documented on this protein (Makela *et al*, 1989; El Kasmi *et al*, 1998; Beauverger *et al*, 1994).

This protein is present on the surface of virus-infected cell and virions as a disulfide-bridged homodimer (Alkhatib and Briedis, 1986). The H protein is folded and dimerized in the endoplasmic reticulum (Bellini *et al*, 1983; Malvoisin and Wild, 1993). It has 13 highly conserved cysteines in the extracellular domain that form intra- and intermolecular disulfide bonds. These are critical for maintaining the three-dimensional structure of the protein, and thus its antigenic characteristics (Hu and Norrby, 1994). There are 5 predicted glycosylated positions in this glycoprotein (Alkhatib and Briedis, 1986), but the one at position 215 is heterogeneous. The glycosylations play an important role in the folding, dimerization and export from the Golgi apparatus as well as for the antigenicity of the protein (Griffin, 1996).

Both hemagglutination and hemadsorption are properties of the H protein (Takenhara *et al*, 1992; Varsanyi *et al*, 1984). Strains of MV that vary in hemagglutinating ability have been described. Many recent wild-type MV isolates have low hemagglutination ability (Saito *et al*, 1992) and many of these viruses encode an H protein with an additional glycosylation site at residue 416 (Saito *et al*, 1995; Saito *et al*, 1994).

I.4.4.2 The fusion protein (F protein)

The F protein is another surface glycoprotein of the MV. It is a type I membrane protein constituted of 553 amino acids, anchored in the viral membrane by a C-terminal hydrophobic domain. Its sequence is highly conserved. The protein is synthesized as a biologically inactive polyprotein of 60kd. After translation, the F protein is glycosylated, which is necessary for further processing, tetramerized and transported to the plasma membrane. In the trans-Golgi compartment, the protein is cleaved by a protease of the host-cell to generate a 41kd F1 and an 18kd F2 disulfide-linked mature F protein (Hardwick and Bussel, 1978). The N-terminus of F1 contains a highly conserved sequence of hydrophobic amino acids. This region is postulated to mediate virus fusion with the cell. This process can also be blocked by F-specific virus neutralizing antibodies. Fusion occurs preferentially when the H protein is present (Schmid *et al*, 1992; Taylor *et al*, 1991a; Wild *et al*, 1991) and F1 seems to be interacting with H using a cystein-rich region (337-381). The F protein plays also a role in viral budding (Cattaneo and Rose, 1993).

While both surface glycoproteins, H and F, induce virus-neutralizing antibodies, the fusion protein seems to be considerably less immunogenic. The F protein is easily accessible to humoral immunity but also to an MHC-II restricted response as is apparent from large amount of CD4+ epitopes found on this protein (Muller *et al*, 1995; Partidos and Steward, 1990; Partidos *et al*, 1991; Partidos and Steward, 1992; Obeid and Steward, 1994; Steward *et al*, 1995; Obeid *et al*, 1995; Atabani, 1997).

I.4.4.3 The nucleoprotein (N protein)

The mRNA of the nucleoprotein is the first and most efficiently transcribed protein. The N protein is also the most abundant of the measles proteins. The N protein is synthesized on free ribosomes and folded in the cytoplasm (Gombart *et al*, 1993). It self-assembles into the nucleocapsid containing the RNA (Fooks *et al*, 1993) but usually surrounds viral genomics and messenger RNA possessing the leader sequence (Castaneda et Wong, 1990) to form the nucleocapsid. This structure is the required template for both replication and transcription and constitutes the ribonucleid complex with the P and L proteins (Ray and Fujinami, 1987). Due to its abundance in infected cells, the N protein is a major antigen of the measles virus, although it does not generate neutralizing antibodies and contributes only indirectly to virus clearance. Its intra-cytoplasmic function and origin triggers MHC-I type of response and a number of CTL-epitopes are known on this protein (Nanan, 1995; Beauverger *et al*, 1993).

I.4.4.4 The phosphoprotein (P protein) and the C and V proteins

The P protein is a 72kd phosphorylated protein that binds to the nucleoprotein and the RNA. The P protein also takes part in the replication complex (Huber *et al*, 1991; Robbins and Bussel, 1979; Stallcup *et al*, 1979). The P protein is very sensitive to proteolysis and is found in limited number in the packaged virus despite its abundance in the infected cell. The P gene codes also for two other proteins, the C and V proteins. The C protein is encoded by the same mRNA using a different AUG codon and overlapping reading frame (Bellini *et al*, 1985). The V protein shares the initiator methionine and the amino-terminal 231 amino acid residues of the P protein but has a shift in the reading-frame due to an extra non-template-directed guanosine residue at position 751, resulting in replacement of the last 276aa of the P mRNA with a cysteine-rich sequence of 68 residues (Cattaneo *et al*, 1989) with zinc-binding properties (Liston and Briedis, 1994). The function of C and V protein in uncertain but they are required for efficient MV replication indicating that they may play a role in the regulation of transcription and/or replication. The immunological role of the P, C and V proteins is minimal.

I.4.4.5 The matrix protein (M protein)

The matrix protein constitutes the major protein of the envelope. M interacts with the F and H transmembrane proteins by several conserved hydrophobic regions. In infected cells, the M protein are associated with the nucleocapsid and with the inner layer of the plasma membrane, suggesting that it may play a role in the viral budding by stabilizing or organizing the membrane environment (Tyrell and

Ehrnst, 1979). The M protein elicits only small amounts of antibody, except in atypical measles (Graves *et al*, 1984; Machamer *et al*, 1980) and has only a minor immunological role.

I.4.4.6 The large protein (L protein)

The large protein is a 2213aa protein is highly homologous to the polymerases of other negative stranded viruses (Blumberg *et al*, 1988). It is present in small quantities in the infected cells and is associated with the viral nucleocapsid in the cell and in the virion. Little is known about the immunological role of L but it is probably minimal.

I.4.5 Main target cells and receptors

CD46 and the newly discovered CDw150 molecule are the two identified cellular receptor for measles virus (Naniche *et al*, 1993; Tatsuo *et al*, 2000).

The CD46 (also know as Membrane Cofactor Protein or "MCP") is a 55-67kd type I transmembrane protein that binds to the complement's proteins C3b and C4b to permit their degradation by Factor I.

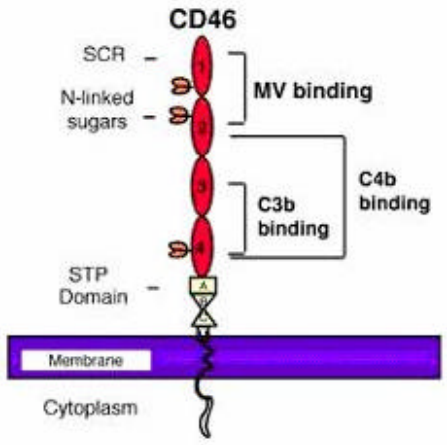

Fig 8: Structure of CD46

This cell receptor is present on hematopoietic and non-hematopoietic nucleated cells. Its cellular function is to set a protective barrier threshold against inappropriate complement activation and deposition on plasma membranes, especially by the alternative pathway of complement activation, by limiting formation and function of the C3 convertases (Liszewski *et al*, 1991). The expression of CD46 is downregulated by infection with some MV strains (vaccine strains) but not by others (wild type strains). This suggested that CD46 is the main receptor for the vaccine-like strain but not for the wild type viruses (Gerlier *et al*, 1994; Schneider-Schaulies *et al*, 1995a, b; Buckland and Wild, 1997). Crosslinking CD46 downregulates IL-12 production. This may be especially important in the immunosuppressive sequelae of measles infections (Liszewski *et al*, 1996). Different isoforms (by

alternate exon splicing plus glycosylation) exists but no distinct functions have been attributed to these tissue-specific isoforms (Johnstone *et al*, 1993). In human, CD46 is found on salivary gland ducts and kidney ducts, moderately on lymphocytes and endothelium, weakly on interstitial tissues and muscle cells. Its expression is often increased on tumor cells and CD46 is not expressed on human erythrocytes (Gerlier *et al*, 1995). A CD46 homolog has been identified in African monkeys but mice do not seem to have a similar protein (Liszewski and Atkinson, 1992; Naniche et al, 1992; Nickells and Atkinson, 1990).

CDw150 (also known as Signalling Lymphocytic Activation Molecule or "SLAM") is a membrane glycoprotein expressed on lymphocytes and dendritic cells, and plays an important role in lymphocyte activation (Tatsuo et al, 2000; Yanagi, 2001). A binding assay with a soluble form of measles virus H protein demonstrated that B-cell lines, activated with Epstein-Barr virus, or T cells, transformed with human T-cell leukemia virus, exhibit this receptor on their cell surfaces. On the other hand, resting lymphocytes, monocytes, or immature leukocytes either failed to express or possessed reduced levels of this receptor (Hsu *et al*, 2001). After infection of cells, and after surface contact with MV envelope proteins, SLAM is downregulated from the cell surface of activated PBL and cell lines (Erlenhoefer *et al*, 2001). Some authors suggest that measles viruses in patients use SLAM but probably not CD46 as a cellular receptor (Ono *et al*, 2001). Furthermore, most measles, canine distemper, and rinderpest virus strains examined could use any of the human, canine, and bovine SLAMs to infect cells. This data suggest that the use of SLAM as a cellular receptor may be a property common to most morbilliviruses and may explain the lymphotropism and immunosuppressive nature of morbillliviruses (Tatsuo *et al*, 2001).

Other possible candidate receptors have been identified on cells of hematogenous, epithelial, fibroblastic and neural origin. These candidate receptors include 20 kd and 30.6 kd proteins on Vero cells identified with an anti-idiotipic antiserum (Krah and Choppin, 1988) and a substance P-binding protein on neurons (Harrowe *et al*, 1990). Moesin (a membrane-organizing extension spike protein) on human leukocytes and astrocytes cell lines was thought to be one of the candidate receptor (Dunster *et al*, 1994) but appeared to be an experimental artifact.

I.4.6 Cycle of the virus

As mentioned before, measles is mainly transported by respiratory droplets ejected from the respiratory tract from infected individuals. These droplets will penetrate the lungs of the naïve individuals during the respiration cycle. The infection starts with the adsorption of the virus on tracheal and bronchial epithelial cells (Sakaguchi *et al*, 1986; Sherman and Ruckle, 1968) and pulmonary macrophages (Kamahora and Nii, 1961; Sergiev *et al*, 1960) using the H protein. Viral infectivity requires maturation of the F glycoprotein probably induced by a conformational change of the H protein

after adsorption. This transmembrane protein initiates fusion with the plasma membrane as a means of introducing the viral genome into the cytoplasm. The nucleocapsid is thus liberated into the cytoplasm of the target cell (Lecouturier *et al*, 1996).

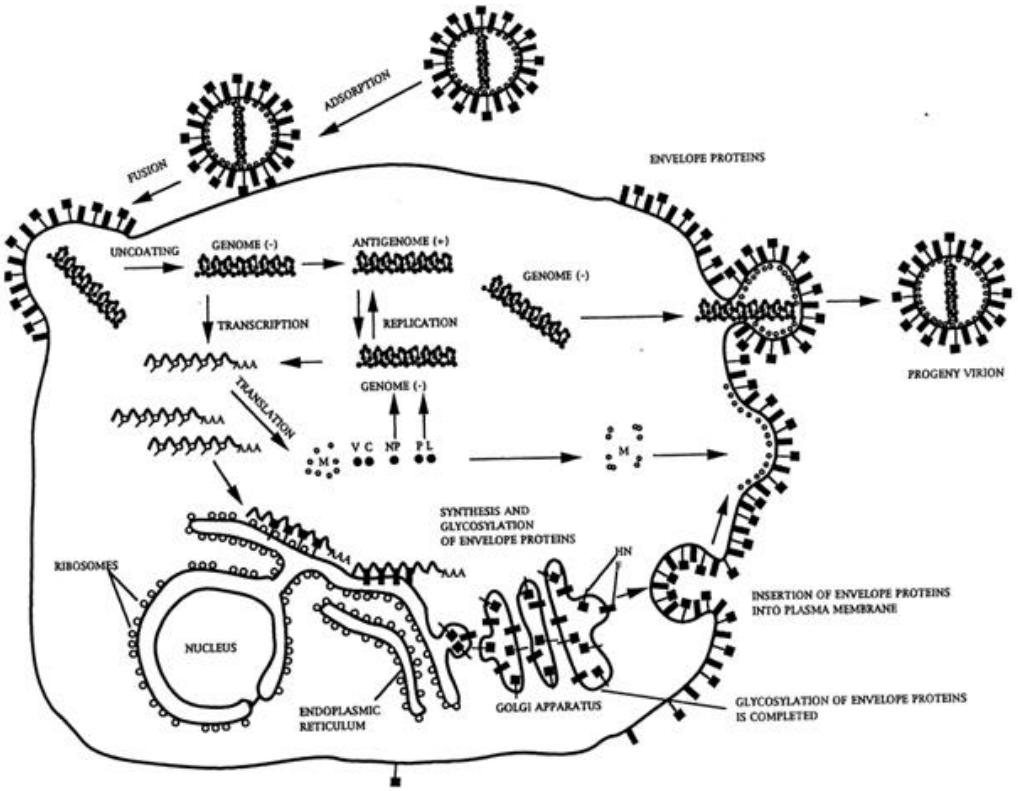

Fig 9: Cycle of measles virus

The genomic RNA is tightly wrapped around the N protein to form a helicoidal structure to which the P and L proteins are attached. This ribonucleoproteic complex (RNP), and not the naked genome RNA, is the template for both transcription and replication. Upon infection, the RNP complex is released into the cytoplasm of the host cell and transcription of viral genes occur using endogenous NTPs as substrates. The product of the L gene, the RNA-dependant RNA-polymerase, plays a key role in transcription and replication. Although it is distributed along the RNP complex, the polymerase can initiate RNA synthesis at the 3'-end of the MV genome only. Viral mRNAs are capped and polyadenylated. Each gene is flanked by untranslated 3' and 5' regions. Between the gene-end boundaries are intergenic regions which contain exactly 3 nucleotides. During transcription, the polymerase skips these intergenic regions and re-initiates synthesis at the downstream promoter. Since the polymerase may fall off the RNA template after transcription termination of any gene, transcript initiation at the next ORF occurs less frequently. Accordingly, there is a gradient in the

relative abundance of the different mRNAs, which follows the order of the genes from the 3' to the 5' end of the viral genome. The re-initiation frequency is estimated to be about 50% but more pronounced gradients have been observed in the case of persistent infections of the human brain. Moreover, in these patients the P-M gene junction may be specifically ignored by the polymerase, thus resulting in the production of bicistronic mRNAs. As only the first ORF is translated, this phenomenon leads to the down-regulation of M gene expression. Translation of the N, P, L and M proteins takes place in the cytoplasm whereas the H and F surface proteins are synthesized in the endoplasmic reticulum and undergo a conformational maturation before being transported to the surface. Surface expression of these proteins induces cell fusion with non-infected cells leading to the formation of giant multinucleated cells.

I.4.7 MV genome replication

Following primary transcription and translation of viral genes, the polymerase switches to a processive mode and ignores the gene junctions to synthesize a full, complementary strand of genome length. This positive-stranded RNA (antigenome) does not serve as a template for transcription and its unique role is to provide an intermediate in replication. The intracellular concentration of the N protein is thought to be the main element controlling the relative rates of transcription and replication. When N is limiting, the polymerase would be preferentially engaged in transcription, thus leading to an increase in the intracellular concentration of viral proteins, including N. When N levels are high enough to allow encapsidation of the RNA chain, the polymerase would switch to a replication mode. Since encapsidation and genome synthesis are concomitant, a helicase-like activity responsible for the separation of the neosynthesized RNA strand from the template must exist. The polymerase acts in concert with the N and P proteins, as indicated by the observation that both proteins are strictly required for *in vitro* transcription and replication. Conversely, C and V proteins are not essential components of the replicative complex. They are supposed to play a role in the regulation of the switch between transcription and replication.

After synthesis of viral RNA, the (-) genome is rapidly associated with the nucleoprotein, N. The N protein appears to provide the genomic RNA with an appropriate configuration for transcription and replication. The N protein is then associated with the P and L protein to form the nucleocapsid. This structure moves to the plasma membrane where it is associated with the M proteins. Electron microscope studies reveal the genome associating with the viral matrix proteins at the plasma membrane. This region of the membrane appears thickened, due to the presence of H and F proteins. From here, the virus is released from the cell by budding. Viral replication takes about 24 hours (Plotkin and Mortimer, 1992; Lamb *et al*, 1996)

During 2-4 days, MV replicates locally in the respiratory mucosa. After this initial period, there is an extension of the infection to the local lymphatic tissues, perhaps carried by pulmonary macrophages (Kamahora and Nii, 1961; Sergiev *et al*, 1960). MV replication in lymphoid tissue results in the appearance of lymphoid or reticuloendothelial giant cells first described by Warthin (1931) and Finkeldey (1931). These are multinucleated cells resulting of the fusion of MV infected cells. These cells are thought to be a major source for spread to other tissues. The amplification of the virus in the regional lymph nodes results in the appearance and spread of the virus through the blood to infect a variety of organs. The primary cell infected in the blood is the monocyte (Esolen *et al*, 1993). MV infection of macrophage cell increase the expression of LFA1, and the cell entry into the tissue is increased (Attibele *et al*, 1993). The late stages of the infection are accompanied by leucopenia.

The lymphoid organs (thymus, spleen and lymph nodes) and the lymphoid tissues (tonsils, appendix) are the main sites of virus replication in the body (Hall *et al*, 1971; Ono *et al*, 1970; Sakaguchi *et al*, 1986) although MV also spread into the skin, conjunctivae, kidney, lung, gastrointestinal tract, liver, respiratory and genital mucosa. In these sites, the virus replicates primarily in endothelial cells, epithelial cells, monocytes and macrophages. The maculopapular rash is generated by the infection of the dermal endothelial cells (Blake and Trask, 1921; Kimura *et al*, 1975a; Kimura *et al*, 1975b) followed by the spread of the infection into the overlying epidermis with infection of epithelial cells in the *stratum granulosum* leading to focal keratosis and edema. Epithelial giant cells form and a perivascular mononuclear infiltrate accumulates (Denton, 1925; Ewing, 1909). Koplik's spots are pathologically similar, with involvements of submucous glands (Denton, 1925).

I.4.8 Cytopathic effects

Under a microscope, MV replication in cell culture monolayers of permissive cell lines results in cytopathic changes (Enders *et al*, 1957). The formation of multinucleated giant cells results from fusion of infected cells with uninfected cells. Infected cells also may change from a normal polygonal shape to a stellate, dendritic or spindle-shaped appearance with increased refractivity to light (Milovanovic *et al*, 1957). Both spindle-shaped cells and syncytial cells may contain intracytoplasmic and intranuclear inclusion bodies. Cytoplasmic inclusions contain RNA encapsidated with N protein and covered with P and M proteins (Bohn *et al*, 1990; Robbins *et al*, 1980).

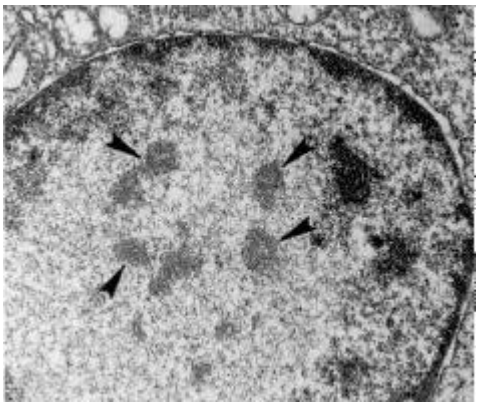

Fig 10: Electron micrograph of a hamster brain infected with an SSPE strain of MV. The arrows show nucleic inclusion bodies.

I.5 Immunity to measles

I.5.1 Immune response to the Virus

The immune response to MV is important for clearance of the virus and recovery from infection. Both the humoral immunity (Black, 1989) and the cellular immunity (Uytdehaag *et al*, 1994) are necessary to stop measles infection.

After the early infection of the lungs, the innate immunity will try to control the infection. NK cells will lysate the infected cells although they are downregulated during MV infection. The abrogation of the NK response may be another element explaining the observed immunosupression during measles infection (Griffin *et al*, 1990b). Macrophages can even phagocyte free MV and destroy it.

The peripheral blood mononuclear cells (PBMCs) start to proliferate (Besser *et al*, 1967; Ward *et al*, 1990), and local inflammatory effect induced by cytokines increase the presence of leukocytes in the tissue, due to vasodilatation and adhesion molecules expression. Polyclonal activation of B cells occurs (Griffin *et al*, 1985) and activation antigens are expressed on T cells (Griffin *et al*, 1986). The concentration of cytokines and soluble cell surfaces proteins also increases (Furukawa *et al*, 1992; Griffin *et al*, 1989; Griffin *et al*, 1992).

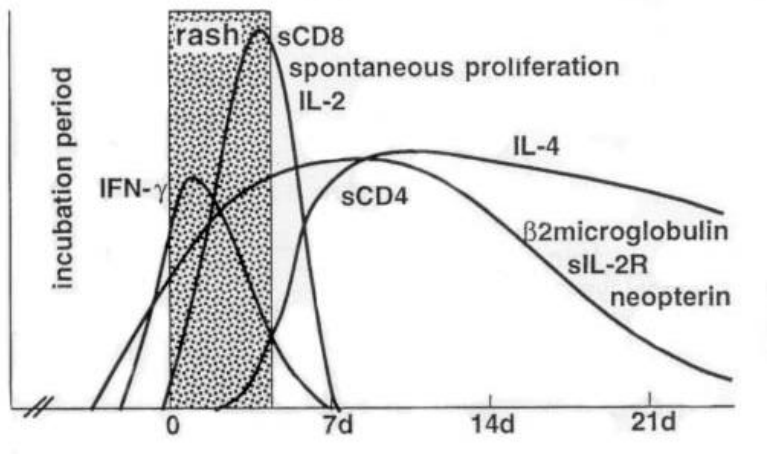

Fig 11: Pattern of cytokines changes in plasma during uncomplicated measles.
These patterns suggest early CD8+ T-cell activation followed by th2 CD4+ T-cell activation.

Production of TNF-α, IL1 and IL6 by the macrophage induces prodromal fever. Cell infected by MV will also produce interferons that will induce anti-viral state (Jacobson and McFarland, 1982; Volckaert-Vervliet and Billiau, 1977). INF-α and INF-β production seems to be induced by the presence of double-stranded RNA in the cell. Such interferons seems to inhibit the viral replication due to the activation of endoribonucleases that degrades viral RNA and inhibit translation along with an overproduction of MHC-I molecules, TAP proteins and proteasomes components, enhancing the capacity of the cell to present viral antigens to CD8+ T cells (Dhib-Jalbut *et al*, 1993). INF-α and INF-β also further activate NK cells.

After an initial replication phase, MV spread to the local lymph node, probably transported by an infected macrophage, or caught by resident cells, where the activation of T and B cell will occur. The ability to recover from measles was postulated by Burnet to be an indication of the adequacy of the T-lymphocyte-mediated immune response (Burnet, 1968). Although early demonstrations of cytotoxicity of leukocytes from patients with measles were primarily antibody dependent (Kreth and ter Meulen, 1977; Kreth *et al*, 1979; Whittle *et al*, 1980), there is abundant evidence that CD8+ T cells are activated during infection. MV antigens inducing CD8+ cytotoxic T cells have not been completely identified, but the F protein is one target for MHC-I restricted T cells (Van Binnendijk *et al*, 1993) and several authors have identified CD8+ epitopes on H and N proteins (Beauverger *et al*, 1994; Beauverger *et al*, 1993; Nanan *et al*, 1995).

CD4+ T cells are also activated. Binding of the H protein to CD46 is targeting virion proteins to an endosomal compartment for efficient presentation by MHC-II proteins (Gerlier *et al*, 1994). CD4+ are proliferating during the rash (Ward *et al*, 1990). Classic CD4+ T cell responses, such as MV antigen

specific proliferation can be induced by most MV proteins in immune individuals (Pette *et al*, 1993; Rose *et al*, 1984). Proliferation has been shown to be specific to H, N, P, F and M proteins (Bellini *et al*, 1981; Van Binnendijk *et al*, 1993).

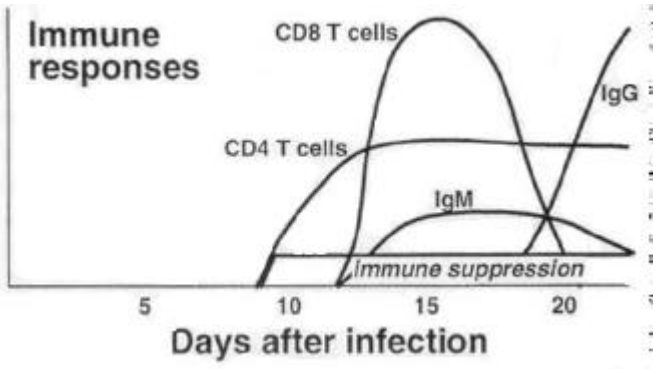

Fig 12: Summary of the humoral and cellular response to normal MV infection

Depending upon cytokines present during the initial phase of CD4+ T cell proliferation, activated T cells become either inflammatory CD4+ (Th1 cell) or helper cell (Th2 cell), determining whether the immune response will be dominated by antibody production or macrophage activation. Th1 CD4+ produces INFγ which activates macrophages and IL-2 which promotes T cell proliferation. Th2 CD4+ produces IL-4, IL-5 and IL10 which are important for B-cell growth and differentiation as well as macrophage deactivation.

During measles infection, the earliest cytokines are INFγ, neopterin (produced by activated macrophages) and soluble IL-2 receptor (Griffin *et al*, 1990a; Griffin *et al*, 1989). This is followed by the elevation of IL-2, soluble CD4 and soluble CD8 at the time of the rash (Griffin *et al*, 1989; Griffin *et al*, 1992). As the rash fades, IL4 becomes elevated and this elevation persists for weeks (Griffin *et al*, 1992). This suggest early activation of CD8+ and type 1 CD4+ during the rash, followed by type 2 CD4+ during recovery.

Full activation of T cell takes 4-5 days and generates changes in the surfaces adhesion molecules to help them to reach the infection focus. Activated Helper T cells will link to a B cell for activation and development of T-cell dependent antibody response. The antibodies only become detectable when the rash appears (Bech, 1959; Graves *et al*, 1984; Hicks *et al*, 1977). The isotype of MV specific antibodies is initially IgM and is detectable 3 days after onset of the rash, but later switches to IgG1 and IgG4 (Ehrnst, 1978; Mathiesen *et al*, 1990; Schluederberg, 1965) that persists for long period of time. Therefore, presence of IgM antibodies indicates an early infection where IgG antibodies refer to recovery from an infection or a secondary immune response following a new contact with an MV antigen.

The most abundant antibodies are produced against the N protein (Graves *et al*, 1984; Norrby and Gollmar, 1972). Antibodies towards F and H proteins contribute to virus neutralization by preventing adsorption and fusion of the virus to the cell (Malvoisin and Wild, 1990; Giraudon and Wild, 1981; Giraudon and Wild, 1985; McFarlin *et al*, 1980).

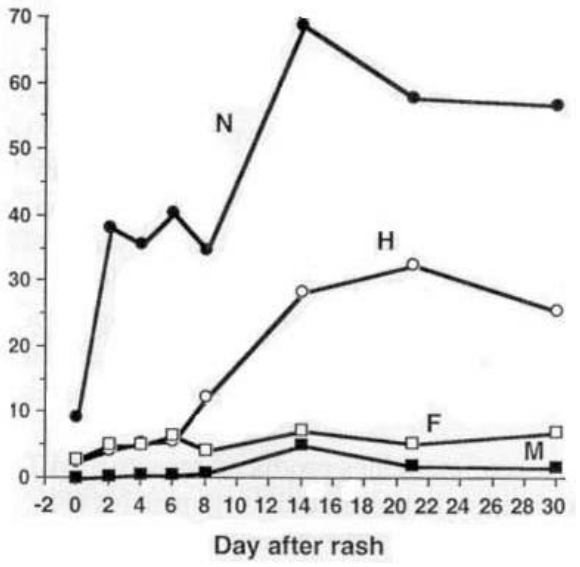

Fig 13: Production of antibodies to measles virus proteins in response to natural infection. Adapted from Graves et al (Graves et al, 1984).

During fusion of the MV with the plasma membrane, the nucleocapsid is injected into the cytosol from where it does not have access to the endosomal pathway, unlike the F and H proteins (Rose *et al*, 1984). Therefore, the N protein in the cytoplasm are degraded and derived peptides are bound and presented by MHC-I molecules. Recent studies have suggested that N protein MHC-I presentation may be TAP independent (Neumeister *et al*, 2001).

At the end of an immune response, when the antigen is cleared, the B cell population decreases, leaving a persistent sub-population of memory cells. Immunological memory is manifest as a heightened ability to respond to pathogens that have been encountered previously and successfully eliminated. It is a property of memory T and B cells, which can transfer memory to naïve recipients (Van Binnendijk *et al*, 1990). One month after contact with the virus, both memory helper T cell and memory B cell can be detected at what will be their higher levels. Immunologic memory includes both continued production of antibody (Black and Rosen, 1962) and circulation of MV specific T cells (Lucas *et al*, 1982; Van Binnendijk *et al*, 1990; Wu *et al*, 1993). The mechanism(s) by which memory cells persist is not fully understood. One theory is that memory cells live for a long time (Mackay, 1993). Another is that memory cells are restimulated at some low level. A number of mechanisms for restimulation have been proposed. Jerne (1974) proposed the idiotypic network theory in which cells

co-stimulate each other in a way that mimics the presence of the antigen. Another theory is that small amounts of the antigen are retained in lymph nodes (Tew and Mandel, 1979; Tew et al, 1980). Another is that related environmental antigens provide cross-stimulation (Matzinger, 1994).

I.5.2 Immune suppression

There is evidence of *in vitro* and *in vivo* immune suppression in measles. Production of antibodies and cellular immune response to new antigens are impaired (Coovadia *et al*, 1974; Whitley *et al*, 1982). *In vitro*, lymphoproliferative responses and lymphokines production are abnormal (Archibald *et al*, 1974; Arneborn and Biberfeld, 1983) suggesting that both monocytes and lymphocytes responses are suppressed. Monocytes are infected during measles (Esolen *et al*, 1993) and infection can lead to changes in monocyte function. TNFα production is decreased during infection. *In vitro* experiments with infected macrophages generated similar decrease suggesting that such abnormalities may result directly from MV infection of monocyte/macrophages. Circulating T cells are also decreased during the acute phase of measles (Alpert *et al*, 1984; Dagan *et al*, 1987; Joffe *et al*, 1983; Whittle *et al*, 1978) and it is postulated that it could be due to inadequate production of IL2 (Griffin *et al*, 1986; Ward and Griffin, 1993). IL4 production seems to be increased during *in vitro* infection, contributing to immune suppression by inhibiting Th1 CD4+ T cells (Griffin and Ward, 1993). *In vitro* infection of lymphocytes with MV vaccine strains induces abnormalities of B- and T-cell function, which may be relevant to immune suppression during measles (Lucas *et al*, 1977; Casali *et al*, 1984).

I.6 Measles vaccination

1.6.1 The history of measles vaccination

The first measles vaccines used were viruses inactivated (killed) by physical or chemical means (by formalin inactivation). This vaccine was then primarily a virus with a destroyed genomic RNA that was able to elicit humoral immunity but no cellular immunity due to its inability to generate viral protein production in the target cell. This kind of vaccine was used in the United States from 1963 to 1967 and was then abandoned after numerous reports of atypical measles in vaccinees when they were exposed to the wild virus. This atypical measles was a severe form of measles with unusual clinical features like higher and prolonged fever, severe skin lesions and pneumonitis (Brodsky, 1972; Nader *et al*, 1968; Rauh and Schmidt, 1965; Rod *et al*, 1970) as well as evidence of hemorrhage. Initial studies of the immune response to the killed measles vaccine have shown that the recipients developed antibodies to the H protein but little to the F or N proteins (Norrby *et al*, 1975; Norrby and Gollmar, 1975). However, more recent studies suggest that these observations may not be related to the induction of atypical measles. These recipients had good lymphoproliferative (Krause *et al*, 1978) and delayed type hypersensitivity responses (Fulginiti and Arthur, 1969; Harris *et al*, 1969, Lennon *et al*, 1967). The

more recent studies suggest that inappropriate cellular response causes atypical measles (Polack *et al,* 1999).

The inactivated vaccines were therefore replaced by attenuated vaccines. This vaccine is a live virus with an attenuated infectivity obtained by passaging the virus on culture cell, thus selecting a "variant" adapted to an unnatural target and therefore having lost its initial efficiency in infecting its original target cell. Such a virus spreads much slower in the organism, allowing the immune system to generate a full response before a wider infection occurs. A drawback of such live vaccines is that they represent a risk to immunocompromised patients.

Current measles vaccines based on the Schwartz strain are further attenuated virus preparations derived from the Edmonston strain isolated in the 1950's (Enders and Peebles, 1954). The culture of this virus on chicken cells resulted in a significant attenuation of its infectivity towards human cells.

Standard titer vaccines contain about 1000 infectious units per dose; higher potency vaccines do not increase sero-responses when administered to children aged 9 months or above and even increased mortality of children between 6 and 9 months (Schnorr *et al*, 2001). Other studies have shown that recipients of high titer measles vaccine formulations suffered increased mortality 2-3 years after vaccination (EPI, 1992).

The normal-titer vaccine is highly effective. In developed countries, the vaccine is administered at twelve to fifteen months, when maternal antibodies have waned. In such conditions, sero-conversion rates reach 95 to 98%. In developing countries, where the vaccine is administered at nine months, sero-response rates and clinical efficacy have usually exceeded 85%. Studies have shown that there is lower seroconversion rate if the vaccine is given to children at six months. This lower sero-conversion rate is partially due to the persistence of maternal antibody in children, but even in infants that have no maternal antibodies, the effectiveness of the vaccine is reduced because of the poor response to the live vaccine of immature immune systems.

Current recommendations for routine vaccination are that a single dose of the measles vaccine be administered to children at the age of nine months in developing countries. An early dose at six months may be useful where there is high risk from measles infection followed by a second dose at 9 months (Cutts, 1993). In developing countries, a single routine dose is insufficient for measles control and a second dose is applied at various ages by an increasing number of countries.

Measles vaccine is administered subcutaneously immediately after reconstitution. In many countries it is combined with the mumps and rubella vaccine and can be administered simultaneously with other vaccines. The vaccine must be kept cool and protected from ultraviolet light after reconstitution.

Inhalation of aerosol or dry powder have also been shown to give satisfactory levels of protection (Licalsi *et al*, 2001; Dilraj *et al*, 2000) and bear some interesting perspectives.

The basis of measles control is to achieve high vaccine coverage with a single routine dose of vaccine given in the first year of life. However, because of factors related to the high infectivity of the disease and its epidemiology, this strategy alone is unlikely to provide sufficiently high levels of control except in isolated environments where transmissibility is low or where very high coverage has been achieved consistently for a number of years.

Despite reaching levels of approximately 82% vaccine coverage throughout the world, outbreaks continue to occur, particularly in pockets of low coverage. Therefore routine vaccination progresses have been invigorated by a second dose at 6 or 12 year of age. In addition, national or regional campaigns, with the vaccination of a wide age group, irrespectively of their vaccination status, have made important progress towards measles control.

I.6.2 Limitations of the current vaccine strategy

Although current live-attenuated measles vaccines have dramatically reduced morbidity and mortality, a very high proportion of the population must be covered to eradicate the disease. Reaching sufficiently high levels of vaccination with a single-dose routine vaccination seems very difficult or impossible. Therefore, nation-wide catch-up campaigns have been set-up in particular in developing countries, to vaccinate all children between a wide age brackets (e.g. 9 month to 14 years) independently of their immune status. Such vaccination campaigns would allow immunizing children that either were missed by routine programs or experienced primary or secondary vaccine failure. Individuals with a weak response to the first vaccination may also benefit from a boost of their immunity. But despite all these efforts, outbreaks continue to occur in countries with high vaccine coverage and two-step vaccination campaigns (Gustafson *et al,* 1987). Current re-emergence of the virus (e.g. in South America) raised questions about the possibility to eradicate measles and about the main hurdles towards elimination of measles.

In an unimmunized population, the virus infects susceptibles who develop measles. After clearing the virus, individuals are immune against the disease. In vaccinees, there is increasing evidence that measles is able to survive and circulate in seropositive individuals (Pedersen *et al*, 1989). After re-exposure to measles, seroconverted individuals may even develop mild disease (Edmonston 1990). In addition, individuals that are fully protected against disease may become transiently infected and develop an asymptomatic secondary immune response (SIR) (Pedersen *et al*, 1989; Muller *et al*, 1996a; Huiss *et al*, 1997). This group of person would be the most likely to support viral transmission in the absence of disease. Although the virus has been isolated from the urine of re-exposed individuals which did not develop symptoms (Vardas and Kreis, 1999), sub-clinical virus transmission has not been directly observed.

Another point of concern with respect to the current vaccination strategy is the susceptibility gap in infants. Young children sustain a higher rate of serious complications from measles and increased mortality but persisting maternal antibodies that interfere with seroconversion complicate early vaccination. In developing countries, children have a higher risk of being exposed shortly after maternal antibodies have waned because of higher measles prevalence and more intensive contacts with susceptibles. In addition, waning of maternal antibodies seems to be quicker in low-income developing countries than previously estimated (Muller, 2001) with high rates of seronegativity already at 4 month of age (Hartter *et al*, 2000). In Nigeria, 21% of measles patients develop disease before being vaccinated at 9 month (Hartter *et al*, 2000). The majority of measles-related death (>50%) occurs with the children between 6 and 12 month (Aaby *et al, 1990)*.

Another possible cause of lower vaccine efficacy may be differences in antigenicity of the measles virus. Although the measles virus is serologically monotypic, it is genetically diverse and different clades and genotypes can be distinguished. In contrast; all vaccine strain viruses are derived from genetically similar viruses of the clade A. Thus within a population of vaccinees, the immune response tends to become increasingly uniform, generating considerable immune pressure on wild-type viruses and allowing the possibility of the emergence of resistant strains. A study in progress in our laboratory has shown that the neutralization capacity of hemagglutinin specific monoclonal antibodies (mAbs) was different in their relative hemagglutination and neutralization activity (Klingele, in preparation). In this study, 24 MV representing 6 genotypes from 5 different clades were evaluated. The clade A viruses were neutralized by all mabs whereas some of the wild-type viruses were neutralized by only 60% of a panel of non-redundant H-specific mabs. Moreover, neutralizing mab titers were substantially higher for vaccine strains than those against wild-type strains. These results suggested that there are significant differences in antigenicity between some wild-type viruses and vaccine strain viruses that appeared to make them more resistant to vaccine induced antibodies. In a similar study, up to 75% of the sera of vaccinees did not neutralize some of the currently circulating viruses in vitro (Klingele *et al*, 2000). However, such vaccinees may still be protected by cellular mechanisms (Griffin, 1995), although the viruses may be able to avoid cellular mechanisms similarly to humoral immunity. These data suggest that differences in antigenicity between wild type and vaccine strains may further reduce protection of vaccinated people and infants (protected only by maternal antibodies).

The sum of the effects of primary and secondary vaccine failure and such factors as lower antibody levels due to vaccination, reduced boosting of immunity by circulating viruses, differences of antigenicity between vaccine strains and a possible asymptomatic virus transmission, rises questions about the need of improved vaccination strategies, using new kind of vaccines that could circumvent some of the above drawbacks of the current vaccines. In addition, after 25 years, 50% of the

individuals vaccinated with life-attenuated measles vaccines are though to have lost protective levels of antibodies (Mossong *et al*, 2000). Whether some of these vaccinees may still be protected by a residual T cell immunity is presently unclear. Such populations would require simple vaccines to boost their immunity.

I.6.3 Novel Vaccine candidates and strategies

Current life attenuated measles (MV) vaccines have effectively reduced the morbidity and mortality of measles world-wide and some progress has been made towards a better heat-stability of life-attenuated measles vaccines, however the attenuated vaccines has a number of drawbacks (Table 1) that could be circumvented by novel vaccines strategies fully exploiting the opportunities provided by molecular biology. Research to develop new vaccines has focused mainly on subunit vaccines based on recombinant Measles H protein combined or not with the fusion protein. A number of different experimental vaccines have been explored by different authors (Table 2). These experimental vaccines may be a favorable long-term solution to the emerging problems of the current measles control campaign. Such vaccine may be more stable at ambient temperature, may be more resistant to maternal antibodies and may be more appropriate for revaccinated vaccinees that have lost protection as a result of waning immunity (Obeid and Steward, 1994).

The current research priorities of the WHO with respect to measles include the characterization of the immunobiology of the measles virus infection and immunization. This goal can be reached by studies of the protective immunity responses including the study of humoral and cell-mediated immunity in animals and man, the identification of the B and T cell epitopes of protective antigens and to investigate the immune response and characteristics of the infection during natural MV infection and immunization.

The WHO has given guidelines for the design of new vaccines. Such new vaccines should provide a high sero-conversion rate and a long term protection, along with low vaccine-related side-effects and complications. Such new vaccines should be resistant to maternal antibodies, have a long shelf-life and be stable at ambient temperature, be easily administered and that could eventually combined with vaccines of other diseases.

Other current research concentrates on developing technologies that eliminates the need to reconstitute measles vaccines before use, or on existing or improved vaccines that explore alternative routes of immunization. For instance, revaccination with an oral vaccine would possibly be one strategy since it does not require the involvement of trained health workers and avoid the risks of infection associated with needles injections. The potential of a parenteral/oral prime-booster schedule has been demonstrated in several studies (Schmucker, 1999).

Moreover, the measles eradication requires simple and quick diagnostic tests of measles infection that can be used in a primary healthcare setting and that could distinguish measles from other diseases. The possibility to discriminate between natural and vaccination-induced immunity would also be a great asset to the possibility to eradicate the measles disease.

In this study, the author will first describe characteristics and changes of immunity in a highly vaccinated population and then describe the design of an immunization strategy based on chimeric recombinant proteins of the measles virus in the context of a changing epidemiology.

Table 1: Advantages and drawbacks of life attenuated vaccines.

Advantages	**Drawbacks**
• single dose • life-long protection • high seroconversion rate(>95%) • low complication rate (1:20000)	• heat-sensitive • lack of resistance to maternal antibodies • concern for immunodeficient vaccinees • no discrimination between vaccine and wild-type induced immunity • low titers of transplacentally transmitted antibodies • possibility to revert to Wild-type virulence • All vaccine derived from clade A attenuated vaccines • role and frequency of SIR susceptibles ?

Table 2: Recent studies on experimental measles vaccines

- Recombinant vaccinia virus (NYVAC) (Kovarik *et al*, 2001)
- Recombinant avian pox viruses – fowlpow virus, canary pox (ALVAC) (Wyde *et al*, 2000)
- Recombinant vesicular stomatitis virus (Schlereth *et al*, 2000a)
- Recombinant adenovirus (Fooks *et al*, 1998)
- Recombinant Bacille Calmette-Guerin (Fenelly *et al*, 1995)
- Recombinant *Streptococcus gordonii* (Maggi *et al*, 2000)
- MV glycoproteins in immunostimulating complexes (ISCOMS) (Pedersen *et al*, 1992)
- DNA vaccines (Polack *et al*, 2000)
- Micro-encapsulated live MV (Nechaeva, 1999)
- Epitope-based vaccines (Hsu *et al*, 1996)
- Recombinant measles virus (Moeller *et al*, 2001)

I.6.4 The secondary immune response (SIR)

Measles infection normally results in life-long protective immunity (Panum, 1940). After the introduction of vaccination, measles morbidity and mortality dramatically decreased (Mitchell and Balfour, 1985), but despite high vaccination coverage measles outbreaks occur (Frank *et al*, 1985; Gustafson *et al*, 1987) because the virus continues to circulate in seronegative individuals. In populations with vaccination rates exceeding 99%, outbreaks were observed in individuals with primary or secondary vaccine failure (Mitchell and Balfour 1985; Gustafson *et al*, 1987). However, there is also evidence that measles virus (MV) can circulate in seropositive populations (Pedersen *et al*, 1989; Gustafson *et al*, 1987; Ozanne and d'Halewyn, 1992; Pedersen *et al*, 1992). The characteristics of this transmission are so far only poorly understood. It is reasonable to assume that people susceptible to develop a secondary immune response (SIR) after reexposure to measles are the most likely seropositive candidates to support viral transmission. There is no direct evidence that such a transmission could lead to clinical measles in seronegatives, although isolated cases without apparent contacts may be suggestive of such a mechanism. With an increasing fraction of vaccinated people, anti-MV titers in the general population tend to decrease (Christenson and Böttiger, 1994) and the epidemiological relevance of SIR-susceptibles may increase. It is therefore important to understand the role of SIR-susceptibles for the epidemiology of measles in a world of global vaccination. We have characterized SIR in parents exposed to children with measles during an outbreak in 1996 (Muller *et al.* 1996a). On the basis of the characteristics of SIR-susceptibles, we will estimate the frequency of SIR-susceptibles among individuals with natural and vaccine-induced immunity to define a high-risk population in which asymptomatic viral transmission could potentially be investigated.

Part II

Material and Methods

II. Material and Methods.

II.1 Principles of the experiments

II.1.1 Cohort study on measles immunity

The secondary immune response (SIR) was investigated in the parents of children with confirmed measles.

Definition of the SIR. An asymptomatic secondary immune response (SIR) to MV is defined as a clinically unapparent increase of specific IgG and/or neutralization (NT) and/or hemagglutination (HI) titers, with or without increase in IgM of an individual with a serologically confirmed first MV exposure and a documented re-exposure to MV.

Parents. Sera of the parents of 84 measles patients were drawn by standard venupuncture during the outbreak. In general, the blood of the parents was drawn when the blood of the children was drawn (14 to 59 days after rash. Average 32 days). They were exposed to one or two children with IgM-confirmed measles. None of the children were vaccinated. As all other parents, they reported having had measles before entering school. Although this was not independently confirmed by a physician, they were born at a time (1949-1970) were immunity was acquired by early natural infection. One to seven pre-exposure serum of each of 45 parents (37 mothers, 7 fathers and 1 grandmother, average age of 37.2years, range 26.3-50.5 and one 67 years old) of children with measles are available from the LNS serum bank. Additional blood samples were later drawn from the same parents (in January 1997, March 1999 and January 2001) to monitor the evolution of their immune status.

Further studies were performed on the following cohorts:

Late convalescent donors. Sera were obtained from 324 consecutive out-patients above 25 years of age of the Laboratoire National de Santé which underwent venupuncture for unrelated reasons in December 1995 and January 1996. Characteristics of the volunteers were as follows: 113 males (25-79 years old, mean 48) and 211 females (25-91 years old, mean 38.4). It can be assumed that the vast majority of the persons in this age bracket had measles during their childhood because they were born at a time (1905-1970) were immunity was mostly acquired by early natural infection. Routine vaccination against measles was introduced in Luxembourg only in 1975.

Vaccinated children. In august and September 1996, sera were also collected from 503 children of the Grand Duchy of Luxembourg from a co-ed public and an all-girls private high school (the Lycée R. Schuman, LS, and the Fieldgen, FI) in Luxembourg-City and a co-ed public high school form a small

town (Wiltz, WI). Only children of the first high school year were included and participation was 63, 76 and 86% respectively. Their median age was 12.7 (date of birth between 12/03/81 and 30/10/85). Immunization status was recorded from vaccination documents. 63.2 % (80.3, 80.5, and 59.7%, for the three schools respectively) of children had record (vaccination card) of at least one measles vaccination, 9.7 % (19.7, 10.3 and 3.6%, respectively) of the children were vaccinated twice and 27 % (20, 19.5, 40.3 respectively) had no vaccination records and were not included in the study.

Early convalescents. With the support of the *Direction de la Santé* of the Ministry of Health, Luxembourg, a hot-line (00352-490648) for measles surveillance was installed in the Dept. of Immunology of the Laboratoire National de Santé (LNS). Between March and July 1996, an outbreak of measles occurred in the cantons of Wiltz and Clervaux in the Grand-Duchy of Luxembourg affecting at least 84 patients, mostly children. Single or paired sera were obtained. Only sera drawn 14-59 days after rash were included in this study.

II.1.2 Principles, clonages strategy and expression systems

We have developed chimeric antigens based on selected T and B cell epitopes. To explore their potential for a new vaccine strategy, two different sets of chimeric proteins were developed.

1. One set of constructs was based on minimal epitopes. This strategy uses 15aa long T cell epitope and one 8aa long mimotope mimicking a conformational B cell epitope of the Measles virus fusion protein. The 3 epitopes will be genetically combined to form a set of permutationnal constructs. This should allow to study candidates vaccines for closing the susceptibility gap in young infants.
2. A second set of constructs uses larger viral protein fragments, to produce a candidate vaccine to boost the residual immunity of individuals with waning immunity.

Although our two strategies will use different elements, the main frame will be as follows:

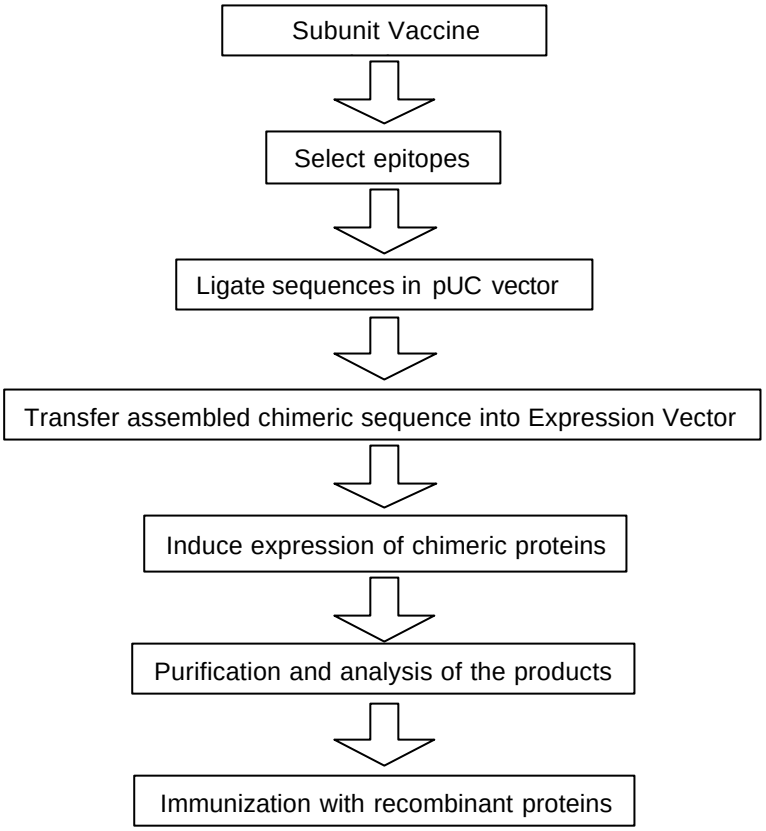

Fig 14: General strategy of design of the chimeric antigens

II.1.2.1 Principle of the clonage strategy

To assemble our constructs into pUC18, we have used a multiple cloning site cassette cloned into pUC18 (seq 400-538) called pUC Start. This cassette contains restriction sites for the isoschizomeric enzymes SalI and XhoI.

The cassettes containing our epitopes were ordered as oligonucleotides (Eurogentec, Liège) with an XbaI and SalI site on its 5' end and an XhoI site on its 3'end. These sequences were then cloned into pUCstart using the XbaI-XhoI sites allowing only one orientation of ligation (5'->3').

The oligonucleotides sequences are extracted using SalI-XhoI digestion. The free ends of the SalI and XhoI sites are compatible (can be religated) but cannot be digested anymore using SalI or XhoI. This system allows serial cloning as described in the Fig15.

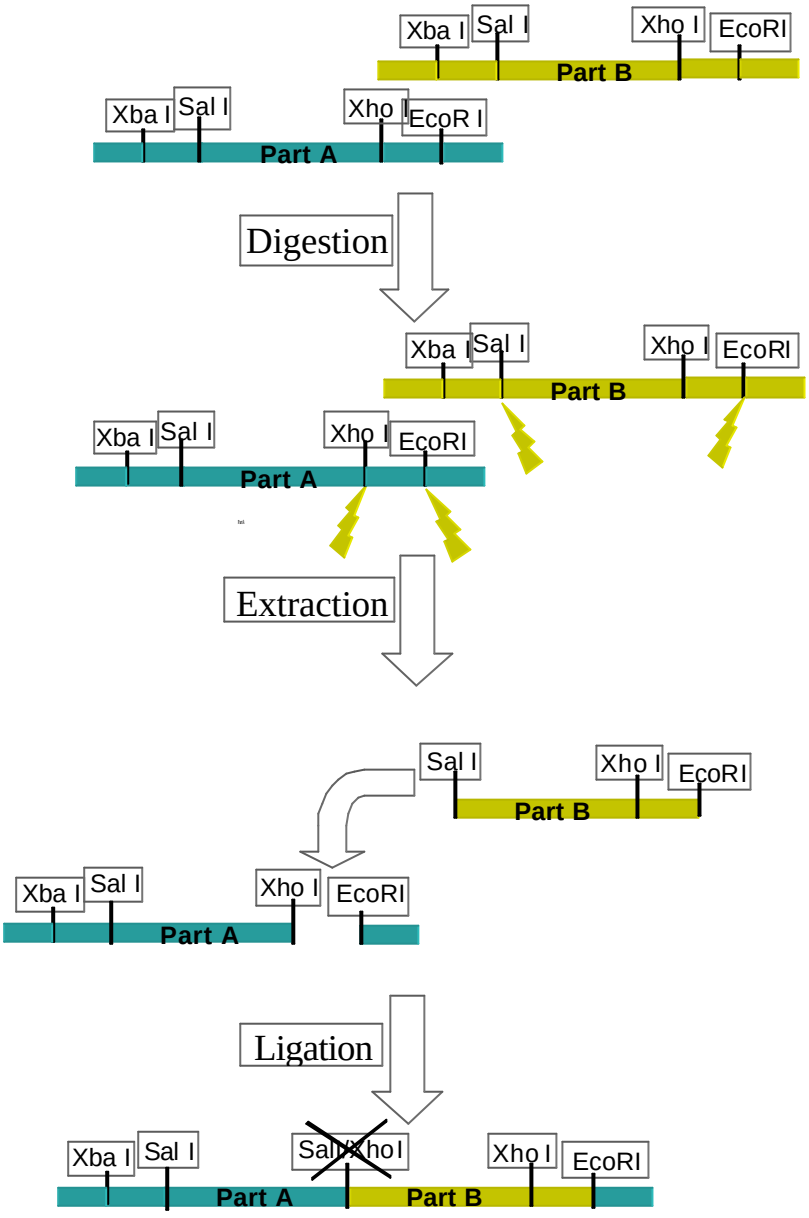

Fig 15: Schematic description of the ligation strategy

In this strategy, a vector containing an instert called A and another vector containing an insert B are digested using the enzymes SalI and EcoRI. The vector A and the insert B are purified and ligated together. The resulting sequence is the contiguous sequence of inserts A and B. The strategy can be reused using SalI and EcoRI or XbaI and XhoI without having the risk of separating A and B since the ligation of the 5' end of B and the 3'end of A is not a restriction site for SalI or XhoI.

Cloning using such system results in the ligation of the two sequences separated by 6 nucleotides (5' CTC GAC 3') coding for Leucine and Aspartic acid. When the constructs are cloned and sequenced, they are transferred into their expression system. The constructs of the "mimotopes constructs" are transferred into pSFV1 using BamH1 digestion and the constructs of the "mv antigenic region strategy" are transferred into pET using NcoI-BamHI digestion.

II.1.2.2 Description of vectors

II.1.2.2.1 pUC vector : The vector used for building our constructs

The pUC vector is derived of the first cloning vectors pBR322 constructed in 1977 by Bolivar and Rodriguez (Bolivar *et al*, 1977). The vector pBR322 was in fact a tripartite replicon consisting of segments from the Col-factor pMB1 (contributing oriV), and from the two R-factors R6-5 (conferring TcR) and R1 (conferring ApR from transposon Tn3). This construct had two resistance markers which could be used alternatively for transformant selection and insertional inactivation.

In 1982 the next generation of cloning vectors emerged constructed by J. Messing and named the pUC series (Messing *et al*, 1982). They were derived from pBR322 by replacing the TcR-gene with the gene for the alpha-peptide of the ß-galactosidase (lacZ') including a multicloning site (MCS) starting at nucleotide 15 (aminoacid 4) of the lacZ' gene.

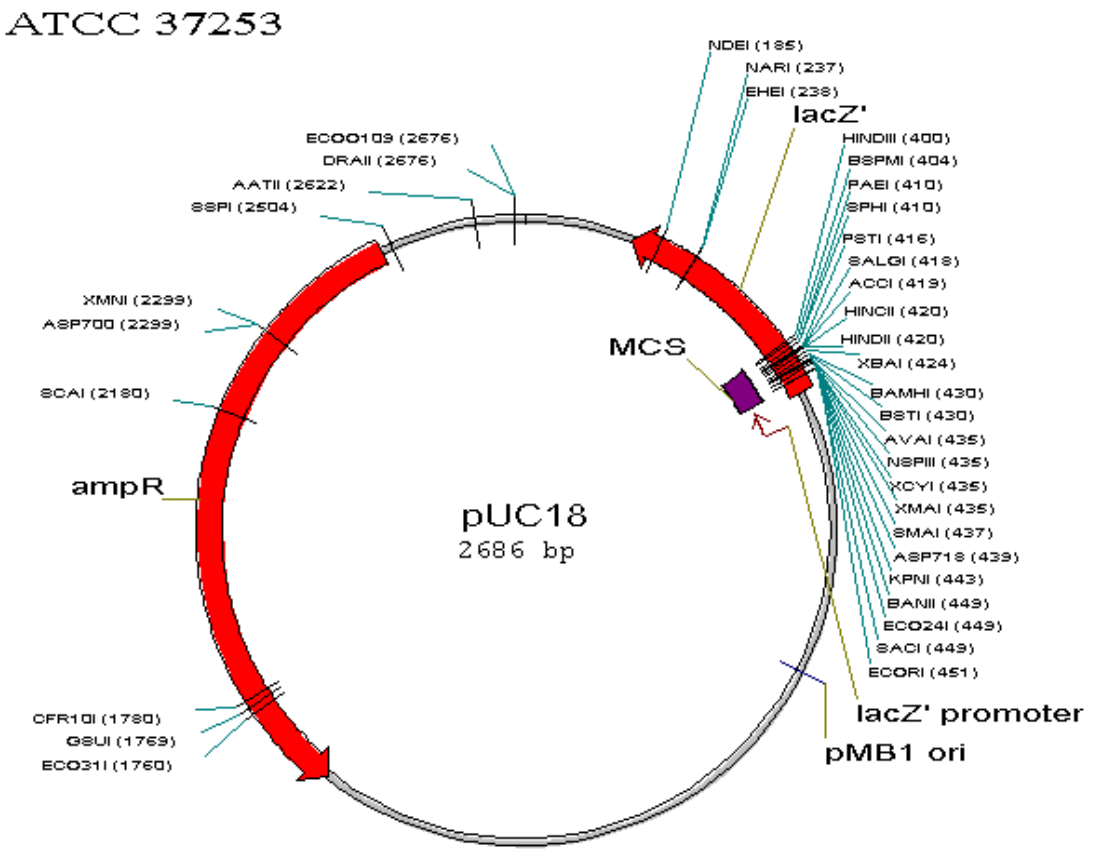

Fig 16: Map of the original pUC18 vector.

In our case, the multiple cloning site of the original pUC18 was replaced by a Start cassette compatible with our whole range of cloning strategies. The fragment HindIII-EcoRI (seq 400-451) of the pET16 was digested out and our cassette (Fig 17) was cloned at its position.

```
    The Start Cassette

    HindIII                              BglI
    |     BamHI              NcoI        SfiI     XbaI          SalI
    |     |                  |           |        |             |
    AAGCTTGGATCCGAGCTCCTGCAGACCATGGCCATTTTGGCCGGTCTAGAGCTAGCGGTCGACACGCGTTGA
400 -+---------+---------+---------+---------+---------+---------+---------+ 470
    TTCGAACCTAGGCTCGAGGACGTCTGGTACCGGTAAAACCGGCCAGATCTCGATCGCCAGCTGTGCGCAACT

                              BglI
              XhoI            SfiI                BglII BamHI EcoRI
              |               |                   |     |     |
    GAAGCGGCCGCCCTCGAGCTTAAGGCCGCTGGGGCCGATAATTAATTGAAGATCTGGATCCGAATTC
471 ---------+---------+---------+---------+---------+---------+------- 538
    CTTCGCCGGCGGGAGCTCGAATTCCGGCGACCCCGGCTATTAATTAACTTCTAGACCTAGGCTTAAG
```

Fig 17: Map of the START cassette Multiple Cloning Site.

II.1.2.2.2 pSFV : The eukaryotic expression system

Several expression systems have been used to study and overexpress cloned eukaryotic proteins. The pSFV gene expression system is a novel expression system based on the Semilki Forest Virus (SFV) replicon that can produce recombinant proteins into eukaryotic cells (Liljeström and Garoff, 1991). In this system, the DNA of interest is cloned directly into the pSFV1 vector that serves as a template for *in vitro* synthesis of recombinant RNA. The RNA can then be transferred into cultured animal cells, or even packaged into conditionally infective, recombinant SFV (Berglund *et al*, 1993). Due to the broad host range of the SFV system, almost any eukaryotic cell type (unlike bacculovirus systems) can be infected with recombinant viral particules. The SFV RNA molecule has a positive polarity and thus functions directly as mRNA. This system has already been used successfully for expressing several proteins in mammal cells (Liljeström and Garoff, 1991).

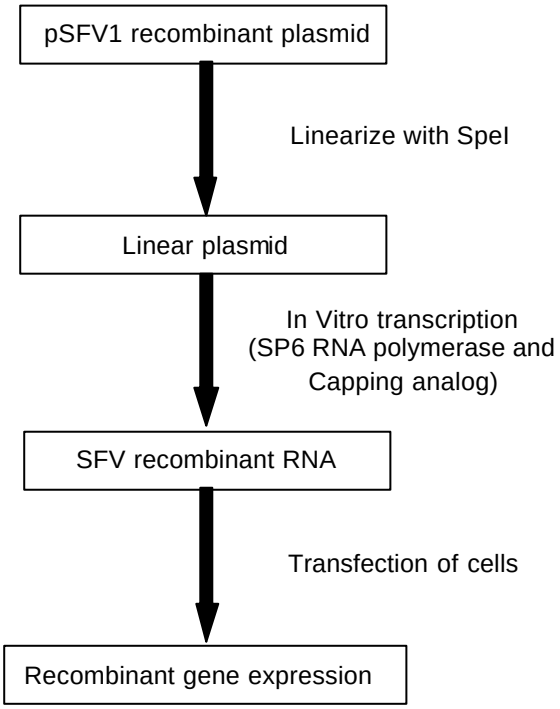

Fig 18: Recombinant gene expression in vitro using pSFV1.

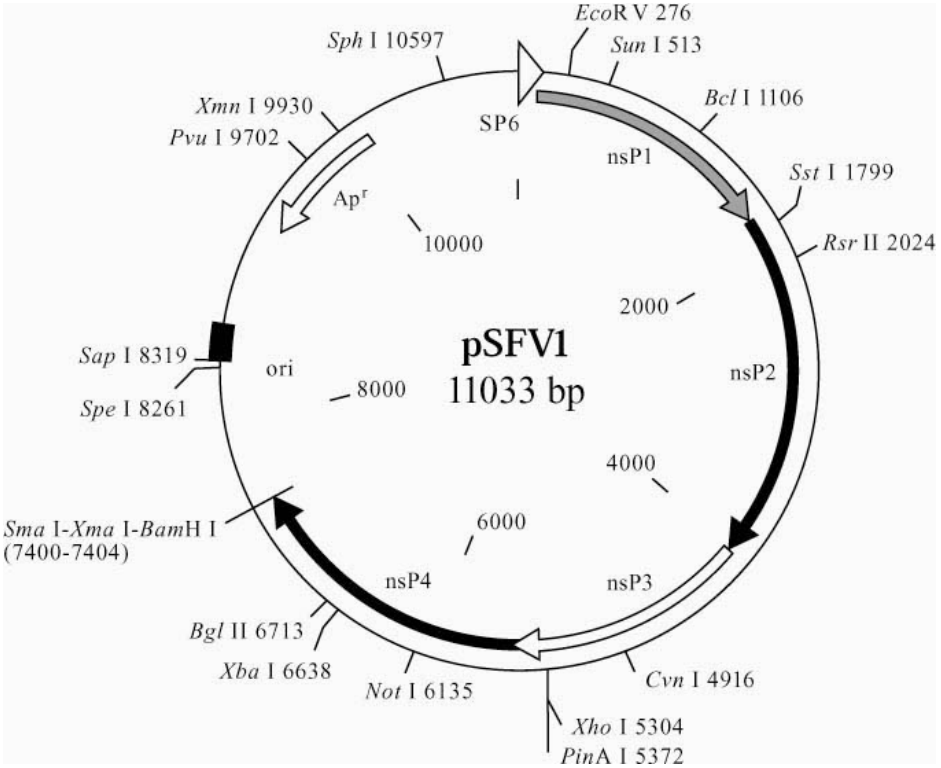

Fig 19: Map of the pSFV1 vector.

```
                                                          BamHI
                                              EcoRI       |   XmaI
      28S Promoter                            |           |   |
pSFV1 ACCTCTACGGCGGTCCTAGATTGGTGCGTTAATACACAGAATTCTGATTGGATCCCGGGT
      ---------+---------+---------+---------+---------+---------+

                                        XmaII
STOP Cassette                           |
      AATAATTGAATTACATCCCTACGCAAACGTTTTACGGCCGCCGGTGGCGCCCGCG
      ---------+---------+---------+---------+---------+---------+
```

Fig 20: Polylinker region of pSFV1

Our constructs are transferred into pSFV1 using the BamHI site at position 7000.

II.1.2.2.3 pET system, the bacterial expression system

For an easier and more efficient expression of recombinant proteins and polyepitopes that do not require mammalian glycosylation, a new set of vectors has been designed. These vectors will express our recombinant proteins into prokaryotic systems, allowing potentially high output and possible long-term conservation of the transformed bacteria.

Our new vectors include a signal sequence for periplasmic expression (pel-B) to ensure the formation of disulfide bridges, necessary for the right conformation of some epitopes used as subunits (our MVH fragments have 3 cysteines at positions 382, 387, 395). They also feature a

His-tag region for easy purification and detection of the recombinant proteins from bacterial supernatants.

The starting point of this system construction is the pET16 vector from Novagen (plasmid featuring a T7 promoter and a lac operator for regulation of its expression), a plasmid for cytoplasmic production in E Coli, originally designed by Studier *et al* (Studier and Moffatt, 1986; Rosenberg *et al*, 1987; Studier *et al*, 1990).

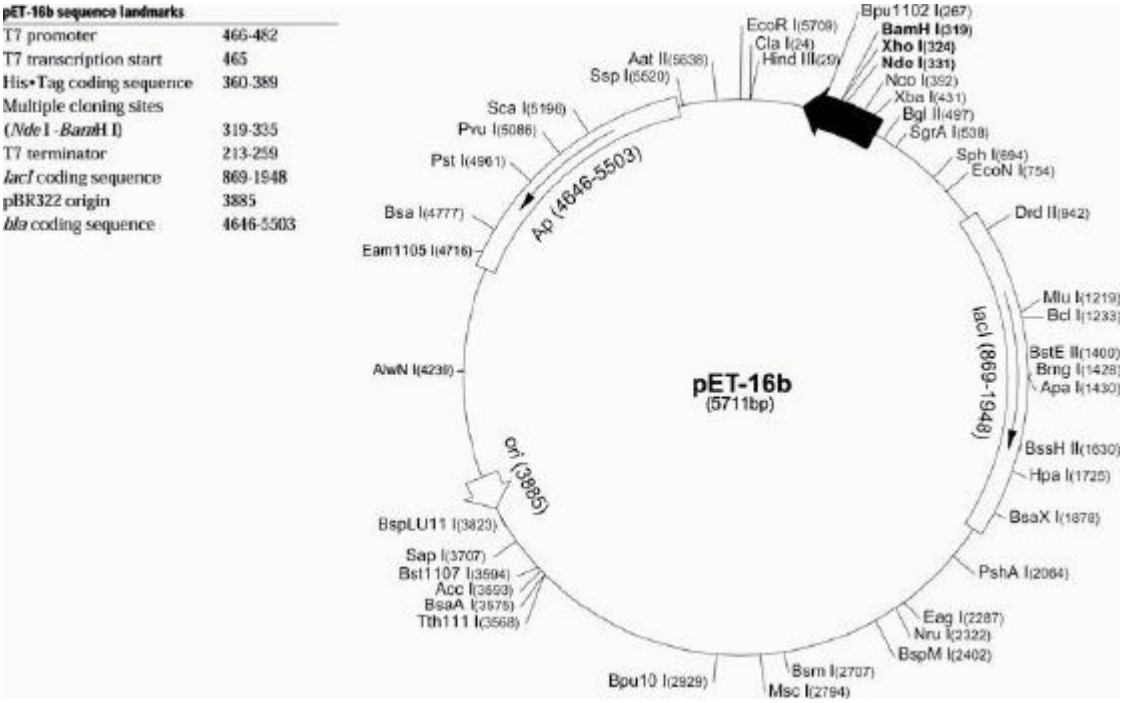

Fig 21: Map of the pET16b vector.

The pET system is used for the cloning and expression of recombinant proteins in *E. Coli*. The target genes are cloned in pET plasmids under the control of the strong promoter of bacteriophage T7. The expression is induced by providing a source of T7 RNA polymerase in the host cell. The T7 RNA polymerase is so selective and active than almost all of the cell resources are converted to target gene expression. The desired product can represent a significative proportion of the total cell protein after a few hours of induction (Studier and Moffatt, 1986, Tabor and Richardson, 1985). Another important benefit of this system is its ability to maintain target genes transcriptionnaly silent in the uninduced state. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell. Once established in a non-expression host, plasmids are then transferred into expression hosts

containing a chromosomal copy of the T7 RNA polymerase gene under LacUV5 control, and expression is induced by the addition of IPTG.

Selected host strain :

The used host cell is the BL21DE3 {genotype: F⁻ ompT hsdS$_b$ (r_b^- m_b^-) gal dcm (DE3)}. A λDE3 lysogen on strain BL21 is the most widely used host for target gene expression. As an *E. Coli* B strain, BL21 lacks the *lon* proteases and *ompT* outer membrane protease that can degrade proteins during purification (Grodberg and Dunn, 1988). Thus, at least some target proteins should be more stable in BL21 that in host strains containing these proteases.

BL21DE3 containing pET recombinants can be kept at -80°C in glycerol 10% stocks. Higher concentration of glycerol can lead to plasmid instability. Expression of the target DNA is induced by the addition of IPTG to a growing culture.

Principle and method of induction

Induction cells must have a chromosomal T7 RNA polymerase gene under LacUV5 repression. The expression is induced by addition of IPTG (isopropyl-D-thiogalactopyranoside) that binds to lacUV5 and prevents repression of the polymerase.

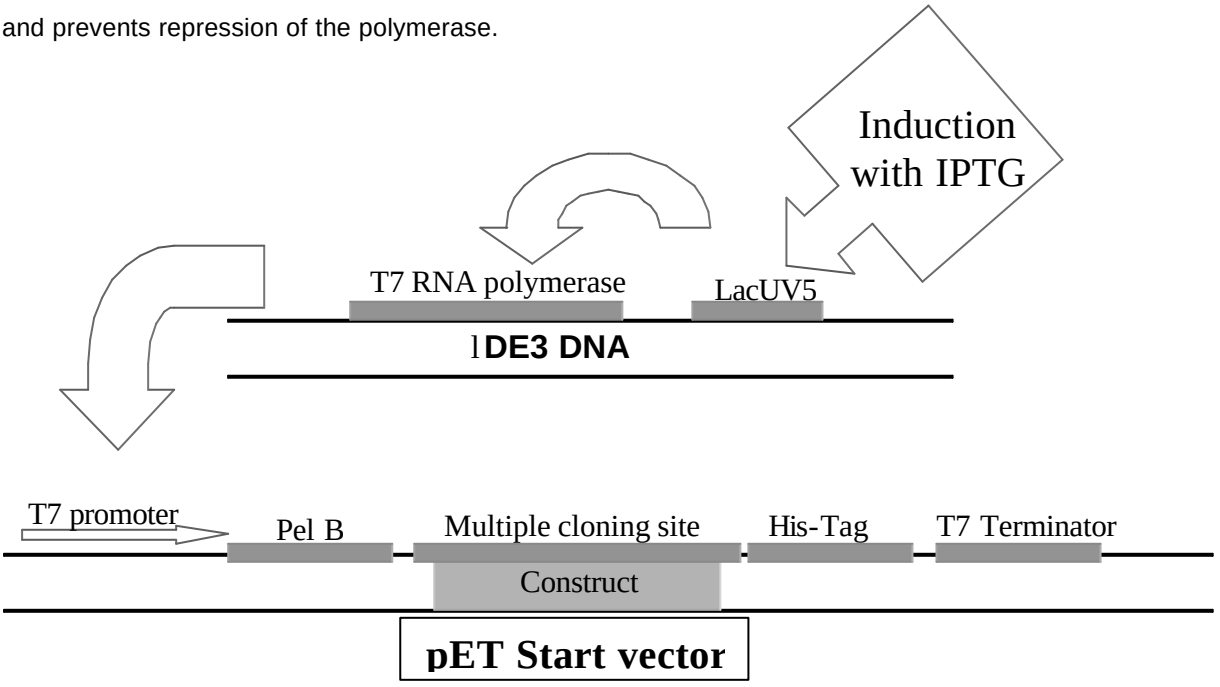

Fig 22: Principle of the expression control of pET vector in BL21DE3 cells

To allow transport of the target proteins to the periplasmic space where dissulfide bonds formation may take place in E-Coli (Derman *et al*, 1993), the pelB leader cassette will be cloned in front of our recombinant genes. Translocation in *E. Coli* is incompletely understood (Wickner *et al*, 1991) but it is known that translocation also depends on the mature domain of the target protein, which is recognized

by SecB, the major chaperone of export. The pelB will be transferred from another vector of the pET family: the pET27 vector, and be ligated in front of pET16 multiple cloning site.

Our vectors have been designed to remain compatible with the pSFV1 eukaryotic expression vector to allow the comparisons of proteins expressed in prokaryotic and eukaryotic cells and with other strategies using BamH1, Xba1–Xho1, Sal1-EcoR1 Bgl1 or Sfi1 for cloning.

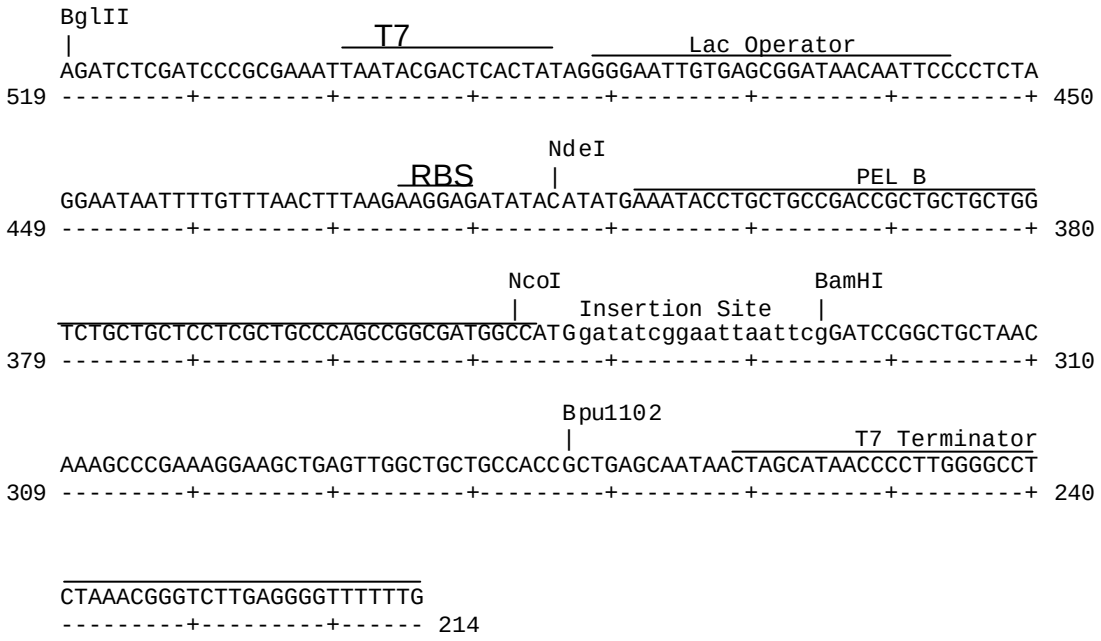

Fig 23: The cassette of our designed pET vector.

Note: The sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map.

Our constructs are finally transferred in pET using the NcoI-BamHI site. Prior to the transfer, the XbaI site in pET16 has been eliminated by cloning a mutated oligonucleotide in XbaI-NdeI.

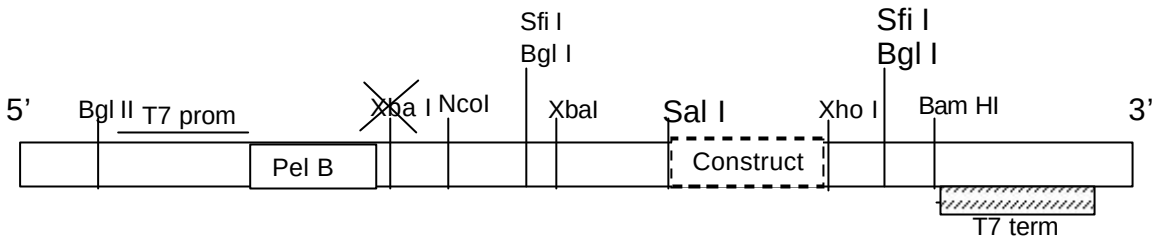

Fig 24: The pet vector MCS with its construct ready for expression.

II.1.2.3 Principle of the purification method

The unknown quantity of our recombinant protein obtained by induction and the need to remove all active bacterial components from our test samples required an extensive purification. We have used the His-Tag region cloned at the end of our proteins to purify them on an HPLC Nickel column. This step would also allow to purify only the complete proteins, since the C terminal His-Tag would be missing on incomplete or cleaved proteins.

Our chromatography column has nickel atoms (Ni^{2+}) stationed inside it. Nickel (and other 2+ metals) is a "transition metal" and therefore is able to bind electron-rich molecules like histidine. Proteins without poly-histidine tail will flow through the column but our his-tag protein will be bound by the Ni^{2+}. A first washing of the column is done with imidazole to release the weak binders (for examples, the bacterial proteins with few histidines). The release of the His-tag proteins is done using higher imidazole concentrations.

II.2 Protocols

II.2.1 Immunology techniques

II.2.1.1 Serum Collection

II.2.1.1.1 Measles Hotline and LNS serum bank

With the support of the *Direction de la Santé* of the Ministry of Health, Luxembourg a hot-line (00352-490648) for measles surveillance was installed in the Dept. of Immunology of the Laboratoire National de Santé (LNS). Between March and July 1996, an outbreak of measles has occured in the cantons of Wiltz and Clervaux in the Grand-Duchy of Luxembourg affecting at least 84 patients, mostly children. Single or paired sera were drawn from the measles patients and their parents by standard venupuncture after informed consent. Sera were aliquoted and frozen/thawed not more than twice before the assays. An additional 110 pre-exposure sera from parents of children with measles were used after informed consent from the LNS serum bank.

II.2.1.1.2 Blood clotting and serum conservation

The analysis of human and mice blood has required extensive blood sampling. The separation of the serum and blood clot had to be carried out carefully to avoid perturbation of the experiments.

The blood was sampled in clean, empty non-heparinized tubes. The blood was heated to 37°C for 30min to allow the blood clot to aggregate. The tube was then centrifugated at 3000RPM for 20min and the supernatant was carefully removed, avoiding to pipet the blood cells. The aliquots of serum were usually transferred into multiples 1.5ml tubes and frozen at -20°C.

II.2.1.2 Serum Analysis

II.2.1.2.1 ELISA

The principle of ELISA (Enzyme Linked Immunosorbant Assay) is to detect an immobilized antigen using antibodies conjugated with an enzyme (usually horseradish peroxydase or alkaline phosphatase) which transforms a substrate into a soluble chromogenic product. After washing away the unbound antibodies, the substrate of the enzymes is added to the antibody/antigen complex and coloration appears. We have used ELISA for the detection of recombinant proteins at key steps of production and purification, or to test the capacity of induced sera to recognize recombinant proteins or complete measles viruses.

The 96 well Maxisorp plates (Nunc) were coated overnight at 4°C with dilutions of protein (max load 300ng/well in coating buffer). The plates were washed 5 times by immersion in washing buffer and the wells were blocked with 200ul of blocking buffer (containing BSA to saturate non-specific binding sites to prevent non-specific background) for 90 min at room temperature. The plates were washed and

filled with 50µl of antibodies in dilution buffer (1:1000 dilution for monoclonal antibodies and 1:100 for polyclonal serum). The plates were incubated 90 min with the first step antibodies, washed and filled with 50µl of a 1:1000 dilution of an alkaline phosphatase-labelled Fc specific antibody (goat anti-Human or goat anti-mouse antibody, Southern Biology Associates). After washing, wells are filled with 100µl of substrate solution.
After 30-60 minutes, the plates can be read in an ELISA-reader at 405nm.

Buffers used for ELISA:

Coating Buffer (pH 9.6): $Na_2CO_3(H_2O)$ 0.1M (Merck) $NaHCO_3$ 0.1M (Merck)	Washing Buffer (pH 8.0): Tween20 0.1% v/v (Sigma) Tris-Base 1mM (Sigma) NaCl 154mM (Merck)	Blocking Buffer (pH 7.4): Bovine serum albumin 1% w/v (Sigma) Tris-Actetate 15mM (Sigma) NaCl 136 mM (Merck) KCl 2mM (Merck)
Dilution Buffer (pH 7.4): Bovine serum albumin 1% w/v (Sigma) Tween20 0.1% v/v (Sigma) Tris-Acetate 15mM (Sigma) NaCl 136 mM (Merck) KCl 2mM (Merck)	Substrate Buffer (pH 10.2): 2-Amino-2-Methyl-1-propanol hydrochloride 1mM (Sigma) $MgCl2.6H_2O$ 0.1mM (Sigma)	Substrate solution: 1 tablet of SIGMA104 (p-Nitrophenyl Phosphate disodium salt) in 10ml of Substrate Buffer.

II.2.1.2.2 Commercial ELISA (Enzygnost)

MV specific IgG and IgM were measured with a commercial ELISA kit based on continuously MV-infected permanent simian kidney cells (Enzygnost, for MV-IgG or IgM purchased from Dade Behring, Marburg, Germany) following the manufacturer's instructions (serum dilution 1:231 for IgG and 1:42 for IgM). For IgM determination, IgG was complexed with sheep anti-human IgG Fc fragment, which enhances the sensitivity and adsorbs rheumatoid factor. Net IgG or IgM (or ΔA) corresponded to the difference in $mO.D._{450}$ between infected and uninfected control wells. According to the manufacturer, ΔA <100 mO.D. or >200 mO.D. are negative and positive respectively. Sera with intermediate values are recommended for retesting, and if not defined are considered "equivocal". Background levels were 80-110 (mean 87) mO.D. for IgG and 70-135 (mean 83) mO.D. for IgM.

II.2.1.2.3 Hemagglutination inhibition (HI) assay

Hemagglutination Inhibition is a diagnostic technique that operates on a principle similar to that of the Coomb's test, except that virus agglutinates monkey erythrocytes. The Hemagglutination Inhibition

allows antibodies to bind to the measles virus before the virus can bind to red blood cells. This type of testing is used in the detection of antibodies to influenza, hepatitis, measles, and rubella. Potency of antibody titers can be obtained using Hemagglutination Inhibition by determining the minimum dilution of serum required to completely inhibit agglutination of red blood cells in the presence of virus.

Triplicates of 2-fold serial dilutions of sera (25 µl/well), were incubated in precooled V-shaped 96 well plates with 25 µl of a standard dose of purified Measles Virus (Edmonston strain, obtained from Dr. Berbers, RIVM, Bilthoven, NL) and 25 µl containing 10^6 African Green Monkey erythrocytes (*Cercopithecus aethiops,*) (Norrby, 1962). All dilutions were done in PBS (pH 7.4) containing 0.2% BSA. HI titer was defined as the highest dilution which completely prevented hemagglutination after 3 h at 37°c. The highest serum concentration tested was 1:22.

II.2.1.2.4 Neutralization (NT) assay

This assay is complementary to the HI test and allows the detection and titration of functionally important antibodies under the same principles than the Hemagglutination inhibition assay. In this case, the titrated antibodies neutralize the virus and prevent it from infecting a monolayer of Vero cells. The antibody titer in the NT test is the highest dilution of serum that still prevents cell infection, monitored by the presence of cytopathic effects (Cell death or presence of giant multinucleated cells).

Two-fold serial dilutions of heat-inactivated serum in DMEM (Dubbleco's Modified Eagle Medium, Life technologies) were preincubated in triplicates for 3h at 4°C with 100 $TCID_{50}$/well of Measles Virus. After adding $6x10^3$ Vero cells per well the micro titer plates were incubated under tissue culture conditions. On day 5, virus infection was monitored using a binocular magnifier and each well was checked for the presence of MV-induced cytopathic effects. Serum concentrations refer to the serum dilution in the presence of MV, before Vero cells were added. NT titer is defined as the highest dilution which prevented virus infection in at least 2 out of 3 wells. Sometimes intermediate values were estimated both for NT and HI titers after replication of the experiment. The highest serum concentration tested was 1:32.

The Vero cells used in the NT assay were obtained from the ATCC and maintained in culture in the lab in DMEM (Gibco) containing 1% Ultroser G (Gibco) and 1% Pennicillin Strepomycin Glutamine (Gibco). Before use, the cells were harvested using 5ml of Trypsin/EDTA (Gibco) and washed in PBS (Gibco).

II.2.1.3 Immunological methods

II.2.1.3.1 Mice T cell proliferation Assay

The principle of a T cell proliferation assay is to induce the proliferation of specific T cell line by using peptides presented by antigen presenting cell. The induced proliferation is measured either by Tritiated thymidine incorporation, or by estimating the amount of IL2 produced using tetrazolium salt.

The T cell hybridomas used in Mice T cell assays are immortalized T cells by fusion with a TCR negative thymoma. Stimulation by antigen can only be measured by cytokine (IL2) secretion. T cell hybridomas are weak responders and need high quantity of peptide (over 10 ng) for stimulation.

This assay allows us to test the efficiency of expression of our T cell epitopes (and thus the expression of the whole construct) *in vitro*. In addition, adding antibodies binding to the B cell epitopes can be used to test whether they would enhance the antigen uptake, by measuring the increase of stimulation of epitope-specific T cell line induced by our proteins.

We have performed a T cell assay using the M12.4.1 cell line as antigen presenting cell, which is a B cell lymphoblastoma (Fournier *et al*, 1996). The T cell lines used are two mouse T cell lines, the TNP79 (stimulated by epitope 330-345 of the MV nucleoprotein) and the TNP408 (stimulated by the epitope 380-395of the MV nucleoprotein).

Due to low sensitivity (compared to human T cell line) of the used lymphocytes, we have enhanced the antigen uptake of our APC, by incubating our constructs with a set of antibodies at a final concentration of 100ng per ml and per antibody.

The antibodies used were:
BH47 (specific of the NE region of the H protein: aa236-255)
BH195 (specific of the HNE region of the H protein: aa379-410)
BNP146 (directed to the 376-395 region of the Nucleoprotein)
BNP176 (directed to the 136-144 region of the Nucleoprotein)

All the cell lines used in this assay have become semi-adherent. To harvest the cells, the culture medium was collected and 10ml of PBS/EDTA were added to the TC flask. The flask was incubated for 5 minutes at 37°C. In the meantime, the collected cell medium was centrifuged for 5 minutes at 1200rpm and the supernatant was carefully discarded. The cells were resuspended in PBS/EDTA then added to the cell pellet and recentrifugated 5minutes at 1200rpm. The obtained pellet was resuspended in T cell medium (TCM).

TCM Culture medium:
RPMI 1640 (Gibco)
10% Fetal calf serum (Gibco)
100ug/ml penicillin (Gibco)
100ug/ml streptomycin (Gibco)
2mM glutamine (Gibco)
Sodium Pyruvate (1mM final) (Additional sugar) (Gibco)
Non essential amino-acids (1:100 dilution) (Gibco)
2B-mercaptoethanol (5µM) (reduction agent) (Sigma)
Hypoxanthine-Thymidine (1:50 dilution) (Gibco)

In each well of a 96well tissue culture quality microtiter plate was added 50µl of a serial (1:3) dilution of our 4 antigens diluted in TCM, 50µl of Antibodies (or a corresponding volume of TCM for the wells without antibodies) and 100µl of a suspension containing 100.000 T cell and 30.000 antigen presenting cell per well.

Specific peptides served as positive controls: the Nucleoprotein derived peptides 2111C and NP379 peptide (sequence from aa 379 to 394 of the N protein) were synthesized by solid phase FMOC synthesis.

After filling the wells, the plate was sent back to the incubator for 24hours, to allow the antigen to be processed by the APC and to give some time to the APC to stimulate the T cells.
After the incubation, the plate was removed from the incubator and frozen at –20°C to kill the T cells. The supernatant of every well was then transferred to another 96 well plate which contains 10000 IL2-dependent T cells (CTLL). The IL2 that was produced during the priming of the T cell line induces the proliferation of the CTLL cells.

After 24 hours, WST1 (Takara biomedicals) was added. WST1 is a tetrazolium salt that is cleaved by mitochondrial dehydrogenase in viable cells to produce fromazan, which has a red color. This red dye is proportional to the number of viable cells in the well and allows the quantification of the IL2 produced by the specifc T cell. Twenty-four hours after adding the WST1, the plates are read at 450nm (optical density subtracted from a reference wavelength at 650nm).

II.2.1.3.2 Human T cell experiment

Amplification in the immune response usually involves proliferation of a particular subpopulation of lymphoid cells that are usually in a resting state. Lymphoid cell responses to antigens are primarily measured by proliferation assays (Kozbor *et al*, 1989).

Measurement of proliferative responses of cultured lymphocytes is a fundamental technique for the assessment of their biological response to the stimuli of our constructs.
This experiment differs from the Mice T cell assay by the measurement of the proliferation induced by the stimulatory agent by the incorporation of tritiated thymidine (^{3}H-Thymidine) into the DNA of dividing cells, a process which is closely related to cell proliferation.

The antigen presenting cell used in this assay is an Epstein-Barr transformed human B lymphocyte, EBV6396 with the MHC-II haplotypes DR1 and DR1103.

The specific T-cells will be stimulated by contact with the antigen-pulsed APC and will proliferate in response to the specific antigen, incorporating ^{3}H-thymidine in their DNA. The cells will be harvested and the proliferation will be measured by scintillation in a β-counter.

In this test, we will use the following T-cell lines:
FR6.6 (Specific of the fragment 254-268 of the F protein)
254T2 (Specific of the fragment 254-268 of the F protein)
NP1.5 (specific of the fragment 185-199 of the Nucleoprotein)
TT830 (Specific of the tetanus toxoïd used as a negative control)

The 4 T-cell lines and the APC cell line were kindly provided by Dr Stephan Demotz from the Institut de biochimie, Epalinges, Switzerland.

Starting with a concentration of 1mg/ml solution in PBS, serial 4fold dilutions of our purified constructs lysate have been prepared. The APCs were washed 3 times in RPMI 1640 culture medium (Gibco) and centrifuged 5minutes at 200G. Before use, the APCs should be blocked to prevent non-specific signal generated by the proliferation of the B Lymphocytes. Therefore, the APC are incubated 30min at 37°C with 100mg/ml of Mitomycine C in 10ml of RPMI1640, which will block the cell cycle by cross-linking DNA. After incubation, the APC are washed 3times using RPMI containing 1% Fetal calf serum (FCS). The T-cell lines were washed once in T-Cell Medium (TCM) to remove the IL-2.

After that process, 50µl of T cell were added to the wells, giving 30000 cells per well for FR6.6, 254T2 and TT830 cell lines and 20000 cells per well for the NP1.5 cell line. Finally, 50µl (25000cells) of APC

were added to every wells. The plates were put back in the incubator at 37°C for 48h. At this time, the ^{3}H-thymidine is added to the wells. The dose added to every well correspond to 0.5 µCi (micro-curie) per well. The plates are put back into the incubator for 16hours.

After the incubation, the wells are washed through absorbent paper (Titertek filter paper, ICN) to get rid of the ^{3}H-tymidine that was not fixed by the cells and to coat the cells on the filter paper. This operation is performed using a Skatron 11025 cell harvester (ICN). The filter papers are dried at room temperature during 5-6hours to let the water evaporate (to prevent quenching and to limit the radioactive waste).

The filter papers are then cut and placed into scintigraphic tubes that are filled with 1ml of scintillation cocktail (Optiphase 'hisafe' 2, Wallac). The tubes (Minis 2000, Zinsser Analytic) are loaded in a β-counter (Rackbeta 1209, LKB) for a counting time of 1minute.

Peptides representing the specific epitopes were used as positives controls: One peptide derived from the F protein (P1760, sequence 256-270), from the tetanus toxoid (P2049) and from the nucleoprotein (P1253, sequence 185-199). Control wells containing the APC or the T-cells alone were also planned, to assess the "background" effect of the cellular components alone (spontaneous cell division, non-induced ^{3}H-Thymidine uptake,…).

II.2.1.3.3 Human T cell experiment with pulsing

After seeing the results of the previous T cell assay, we wondered if some of the questionable results obtained could be caused by the blocking of the cell cycle by the Mitomycine C (to prevent proliferation of the APC). Although short peptides can be directly linked to free MHC molecules on the cell surface, longer peptides requires intra-cellular processing that may require full cell activity. In this T cell experiment, we reproduce a part of the previous T cell assay and we compare it with a similar setting where the APC is pulsed 8hours with the constructs before being blocked by the mitomycine C.

For this experiment, we use the NP1.5 T cell line (specific of the fragment 185-199 of the Nucleoprotein) in conjunction with the EBV6396 APC (DR1, DR1103).

We have the same quantities of constructs than in the previous T cell experiment, namely a serial 4fold dilutions of our purified constructs lysate (ABMVH1, AB NP1-7, MVH1 and CBMVH3), starting with 50ul of a 1mg/ml solution of constructs in PBS. Peptides representing the specific epitopes were used as positives controls (P1760 is the sequence 256-270 from the F protein and P2049 as a negative control of proliferation. Two plates were filled as exact duplicates.

One of the plates was kept at 4°c and the other was filled with 50.000 APC per well and incubated 8hours at 37°C. This time will give to the APC enough time to process and express our constructs to its cell surface.
After the incubation, the plates are washed three times with RPMI1640 (Gibco) and blocked with 100mg/ml of Mitomycine C for 30min at 37°C. Five ml of RPMI medium containing 2.500.000 APC were also incubated with 100mg/ml of Mitomycine C, to be added to the non-pulsed plate.
The APC are the washed 3 times in RPMI/TCM medium (4:1) (The FCS in the TCM medium helps inactivating the Mitomycine C) and centrifuged at 1200RPM (200g) for 5 minutes. The APC can then be distributed in the non-pulsed plate (25.000 APC per plate).

The two plates are then filled with the T Cells (30.000 Tcell per well) and placed into the incubator for 48hours. Reading of results is done as in II.2.1.3.2.

II.2.1.3.4 FACS analysis and Staining

An excellent way to detect if our obtained serum contained anti-MV antibodies was to test them in flow-cytometry against MV-infected WMPT cells. The anti-MV antibodies would then bind to the MV-proteins expressed on the surface of the WMPT cell (as H and F proteins) or intracellular proteins (as the N protein) after cell permeabilization. Detection of bound antibodies will be made possible by the use of FITC-labelled secondary-step antibodies.

The WPMT cells must be harvested using PBS-EDTA (the use of trypsine damages the surface proteins) and washed 3 times in FACS medium at 1200RPM at 4°C. 20µl of serum diluted 1:10 are then added to 80µl of cells (200.000 cells) and incubated 15 min on ice. After the incubation, the cells must be washed 3 times as before to remove all unbound antibodies, being very carefull not to suck the pellet up during the removal of the supernatant. The cells are then resuspended in 100ul of FACS medium containing GaM/IgG FITC-labelled secondary-step antibodies diluted 1:200, and incubated 15minutes on ice. After a last washing step to remove the unbound antibodies, the cell pellet is resuspended in 200µl of FACS medium containing 1µg/ml to allow dead cells discrimination. The analysis has been done on a Coulter Epics Elite ESP.

Facs Medium:
PBS 1X (Gibco)
Bovine serum albumine 1% (Sigma)
Sodium Azide 0.1% (Sigma)

II.2.2 Molecular biology techniques

II.2.2.1 Enzymatic manipulation of nucleic acids

II.2.2.1.1 Minipreparation of DNA

This protocol is designed to allow the isolation of small quantities of DNA from bacterial cells, usually to screen bacterial colonies from a Petri dish. Our method is derived from the alkaline lysis protocol (Birnboim and Doly, 1979; Birnboim, 1983). In this technique, the bacteria are lysed with a solution containing Sodium-dodecyl sulfate (SDS) that will denature bacterial proteins, and NaOH that will denature chromosomal and plasmid DNA. The mixture is then neutralized with potassium acetate that causes the covalently closed plasmid DNA to re-anneal rapidly. The chromosomal DNA and bacterial proteins will precipitate with the SDS and are removed by centrifugation. The plasmid DNA contained in the supernatant is concentrated by ethanol precipitation.

Singles colonies are transferred to 1ml of LB medium using sterile toothpicks. The tubes are incubated 4h to overnight at 37°c then centrifuged at 13000rpm for 5minutes. The supernatant is discarded and the pellet is resuspended (vortexed) with 100ul of ice-cold solution1 containing RNAse (120mg/L), and incubated for 5 minutes. 200ul of solution 2 are added and gently homogenized (to prevent genomic bacterial DNA to be separated from the membrane). After 5minutes of incubation at room temperature, 150ul of solution3 are added and mixed. After 5 minutes on ice, the mixture is centrifuged at 13000rpm for 15minutes. The DNA is in the supernatant and must be precipitated with 1volume of Phenol/Chlorophorm/Isoamly-alcohol (Sigma) and centrifuged 5 minutes at 13000rpm. The aqueous phase is precipitated using 2 volumes of Ethanol 100% and put at –80°c for 30minutes. The tubes are centrifuged for 30minutes at 13000rpm and the pellet is washed with –20°c Ethanol 70%, dried and finally resuspended in 50ul of ddH_2O or TE buffer.

Solution 1:
For 100ml:
5ml Glucose 1M (ICN)
2.5ml Tris-HCl 1M pH8.0 (Sigma)
2ml EDTA 0.5M pH8.0 (Sigma)

Solution 2 (freshly prepared):
200ul NaOH 10M (Sigma)
1ml SDS 10% (Merck)

Solution 3:
60ml KOAc (Merck)
11.5ml Glacial acetic acid (Hanff frères)
28.5ml ddH_2O

II.2.2.1.2 Maxipreparation of DNA

The maxipreparation of DNA uses the same principle as the minipreparation, but adapts it to larger volumes of bacterial culture (up to 500ml) and uses anion exchange resin to purify plasmid DNA from the bacterial lysate. The negatively charged phosphates on the backbone of the DNA interact with the positive charges on the surface of the resin equilibrated in a low-salt buffer. Under moderate salt conditions, plasmid DNA remains bound to the resin while RNA, proteins, carbohydrates and other impurities are washed off. Finally, the plasmid DNA is eluted under high salt conditions. The eluate is ethanol-precipitated and the pelleted DNA resuspended inTE buffer.

The alkaline lysis protocol follows the guidelines and uses the solution of the CONCERT high purity plasmid maxiprep system (Gibco). The purification uses the resin provided in the same kit with the following procedure. The column is equilibrated with the provided equilibration buffer and the DNA lysate is filtered on a nylon filter and poured in the column and the solution will drain by gravity. The resin is the washed twice with 50ml of provided washing buffer. The DNA is eluted using 12ml of elution buffer and collected in 1.5ml Eppendorf tube by fraction of 800ul. 570ul of Isopropanol are then added to the fraction. The tubes are cooled at –80°C for 30minutes and centrifuged at 13000rpm for 30minutes at 4°c. The obtained pellet is washed with 70% Ethanol, dried and resuspended in 100ul of ddH_2O or TE buffer.

II.2.2.1.3 Annealing of oligonucleotides

As our cloning fragments were purchased as short oligonucleotides, the two strands of DNA needed to be assembled to make double-stranded DNA.

Both complementary oligonucleotides should be resuspended at the same molar concentration, using Annealing Buffer. Annealing should perform well over a wide range of oligo concentrations.
Equal volumes of both complementary oligos (at equimolar concentration) are mixed in a 1.5ml microfuge tube. The tube is placed in a standard heatblock at 90 - 95ºC and then allowed to cool to room temperature (or at least below 30ºC) on the workbench. For sequences with significant hairpin potential, a more gradual cooling/annealing step is beneficial; this is easily done by placing the oligos in a water bath or temp block with a slow temperature decrease. The obtained fragments should be stored on ice or at 4ºC until ready to use.

Anealling Buffer (pH 7.5 – 8.0):
Tris 10 mM (Sigma)
NaCl 50mM (Merck)
EDTA 1mM (Sigma)

II.2.2.1.4 Enzymatic digestion

The major tools for manipulating DNA and inserting defined inserts into expression vectors are restriction enzymes. These enzymes recognize short DNA sequences and catalyze specific reactions that cleave DNA molecules at specific sites within or adjacent to the recognition sequence. The enzymatic cleavage is achieved by incubating the DNA sample with the enzyme(s) in appropriate ionic conditions at specific, enzyme-depending, temperatures (most frequently 37°C).

In general, we have used the commercially available buffers provided with the enzyme (Invitrogen). One unit of enzyme (1U) is the amount of restriction enzyme required to cleave 1ug of λDNA in one hour in the optimal conditions.
The reaction protocol requires DNA in H_2O or TE buffer.

A typical DNA restriction reaction mix contains:
0.1µg to 4µg of DNA
2µl of 10x reaction buffer
ddH_2O up to 20ul
1U to 5U/µg DNA of Restriction enzyme

The total volume of the reaction may be increased to fit the quantity of DNA that needs to be digested, but the proportions of the reagents must be conserved.

Two or more enzymes can be added in the same reaction mix if their respective conditions (buffer and temperature) are compatible and that given the max percentage of glycerol in the reaction-mix should not exceed 5% of total volume. If the two buffers are different, the best buffer is the one with the lowest NaCl concentration, provided that no unspecific cleavage occurs under these conditions. If the active temperature of the enzyme was different, the digestion was done with the first enzyme, then the temperature was changed and the second enzyme added.

The mix is then incubated at the recommended temperature (mostly 37°c, 25°C for i.e. SpeI) for 30-60minutes. Increasing the incubation time over 2hours does not improve the efficiency of the assay. The reaction can be stopped by adding 10% of Stop gel loading buffer (SGLB), or by precipitating the DNA using ethanol 95%. After washing in cold 70% ethanol, the DNA restriction fragments are ready for further enzymatic manipulations or electrophoretic separation in preparative agarose gels.

Stop gel loading buffer (SGLB):
For 50ml:
25ml of glycerol (ICN biomedicals)
10ml EDTA 0.5M pH7.5 (Sigma)

0.5g SDS (Merck)
0.05g bromophenol blue (Serva)

TE buffer (10 mM Tris-HCl pH 7.8; 1 mM EDTA):
For 600 ml solution:
0.727 g Tris-HCl (Sigma)
1.2 ml of 0.5M EDTA (Sigma)
ddH20 is added up to 600ml and the solution is autoclaved.
pH must be adjusted to 7.8 using 1 M HCl.

II.2.2.1.5 Dephosphorylation of DNA using CIP

Calf intestine phosphatase (CIP) catalyses the hydrolysis of 5'-phosphate residues from DNA. This phosphate residue is necessary for the ligation between two ends of cleaved DNA.

In our case, we have used CIP to prevent the intramolecular ligation (self-ligation) of Bam-H1 cleaved pSFV1 vector and to improve the efficiency of the ligation with an (non-dephosphorylated) insert.

The protocol of the manufacturer (Takara) recommends for 50ul of reaction:
1 to 20pmoles of DNA termini
0.1U of CIP (Alkaline phosphatase from calf intestine, Takara)
in a buffer composed of:
20mM Tris-HCl, pH8.0 (Sigma)
1mM $MgCl_2$ (Sigma)
1mM $ZnCl_2$ (Sigma)

As a reference, for a vector of 5700base-pairs, 2ug of vectors is equal to 1.35pmole of DNA termini. The reaction takes place at 37°c for 1 hour and is stopped by either 10min at 70°c, or a double extraction with phenol/chloroform.

II.2.2.1.6 Ligations

To covalently link two 5' phosphorylated dsDNA fragments, for example vector and insert, in a cloning experiment, we have used T4 DNA Ligase. The vectors and insert used for the ligation must have been carefully purified (using gel separation) to prevent external DNA fragments to be involved in the ligation.

The Ratio between the vector and the insert is critical to obtain high-efficiency ligation. To increase the cloning efficiency, we usually did the experiment in triplicates using different ratios (usually 1:1, 1:5 and

5:1 in molarity) allowing to find the best vector/insert ratio. The recommended total amount of DNA (vector + insert) per ligation is comprised between 0.1µg and 0.5µg. 10µl of plasmid ligation buffer (Clontech) is added to the DNA solution. 1.2ul on a 10mM ATP solution is the added to the reaction mix, followed by 1ul of T4DNA ligase (Clontech).

The reaction was then incubated in a waterbath at 16° for 30minutes. The efficiency may sometimes be increased by elongating the ligation time up to 12hours (overnight).

At the end of the ligation time, glycogen is added to increase the precipitation yield at low DNA concentrations prior to stop the reaction. The mixture is then precipitated with 2 volumes of 100% Ethanol at –80°c for 1hour or more and centrifuged in a microfuge at 13000rpm for 30minutes. The obtained pellet is then resuspended in ddH_2O and used for electroporation.

For ligations using single restriction site (i.e. ligation of a BamH1-BamH1 insert in a pSFV1 vector), the dephosphorylation of the vector is strongly recommended to reduce the amount of intramolecular ligations.

II.2.2.1.7 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is an *in vitro* method for a site-directed enzymatic amplification of specific DNA sequences. The specificity of the PCR amplification is based on two oligonucleotide primers that flank the DNA segment to be amplified and hybridize to opposite strands.
In our case, we have used PCR to extract the desired epitopes from cDNA of measles proteins and it was also used routinely to test the results of plasmid ligations.

The specificity of the amplification reaction can be improved by fine tuning of the annealing temperature and magnesium concentration. A standard reaction mix for a 25ul reaction includes :

2.5 µl of 10x Buffer (Gibco)
1.5 µl of $MgCl_2$ (Sigma)
1 µl dNTP (Gibco)
DNA template
5-10 pmole of forward primer
5-10 pmole of reverse primer
0.3 ul of Taq polymerase (Gibco)
Sterile H_2o (Rnase/Dnase free) up to 25µl

The cycling conditions described hereafter are generic and could vary following the annealing temperature of the primers and the length of the amplified fragments.

Thermal cycler program.

The programs start when the lid of the cycler has reached 105°c

Step	Temp	Time	Action
1	94°c	for 2 minutes	Denaturation (separation of DNA strands)
2	94°c	for 50 sec	
3	55°c	for 15 sec	Annealing of the primers with the DNA Temperature must be the optimal t° for the primers used
4	72°c	for 15 sec	Elongation time. The time may be increased for fragments bigger than 1000bp
5	-----	----------	The programs restart at step 2 for 29 times
6	72°c	for 6 minutes	Polymerase run-off
7	4°c	Until stop	The cycler cool down to 4°c and stops

II.2.2.1.8 Acrylamide Gel used for DNA separation

Horizontal agarose gels prepared in TAE buffer, are generally used to analyse the size of DNA fragments. The concentration of agarose determinates the resolution of the electrophoresis separation (ranging form 0,8% to 1,5% agarose in TAE). Gels of 8x8 cm (called “Mini gels”) have been used. The DNA samples are loaded into the wells with 1:10 (V/V) of loading buffer.

A current of 100V, 80mA is applied to the gels for 30minutes and the DNA can be visualized on an U.V. light due to the ethidium bromide added into the gel. A well of the gel must always be reserved for a reference ladder (In our case, the 1kB+ ladder from Gibco).

TAE buffer (Tris-Acetate EDTA) : Tris acetate : 40mM (Sigma) EDTA : 2mM (Sigma)	Acrylamide gel (1%): 50 ml of TAE buffer 1% (W/V) of Acrylamide (Bio-Rad) 0,5 µg/ml of Ethidium Bromide (Gibco)
SGLB (Stop Gel Loading buffer) (for 50ml): 25ml glycerol (ICN biomedicals) 10 ml EDTA 0,5M pH 7.5 (Sigma) 0.5 g SDS (Merck) 0.05 g Bromophenol blue (Serva) ddH_2O ad 50ml	

II.2.2.1.9 Phenol/chloroform extraction

Phenol/chloroform extractions are used for the separation of DNA and proteins. The DNA is soluble in the aqueous phase while most of the proteins remain in the organic phase, allowing a quick and safe separation.

An equal volume of buffer-saturated phenol/chloroform/isoamyl alcohol (25:24:1) is added to the DNA solution. The solution is the mixed. Most DNA solutions can be vortexed for 10 sec except for high molecular weight DNA which should be gently rocked. The solution is spun in a microfuge for 3 min and the aqueous layer is carefully transferred to a new tube, being careful to avoid the interface. This washing step can be repeated until an interface is no longer visible in the aqueous phase. To remove traces of phenol, an equal volume of chloroform is added to the aqueous layer and centrifugated for 3 min. The aqueous phase is transferred to a new tube and precipitated using Ethanol 100%.

II.2.2.1.10 DNA purification using silica matrix

The gel extraction protocol hereafter is designed to purify small quantities of DNA from agarose gels. This method is suitable for non-supercoiled DNA only. The protocol uses silica resin (Gibco) to capture and purify the DNA fragment. The gel fragment is dissolved in sodium perchlorate and the DNA is adsorbed on the silica support as described by Vogelstein and Gillespie (1976).

The desired fragment is excised from the acrylamide gel. The size of the gel fragment should be as small as possible, following closely the limits of the desired band. The gel fragment is mixed 1:3 (w/v) with a solution containing Sodium Perchlorate and Sodium acetate (Gel solubilization buffer, Gibco) and heated at 50°C for 10minutes with gentle agitation until the gel is dissolved. Add 1ul of silica resin (Gibco) for every 10mg of gel and incubate another 20minutes at 50°C with agitation. The mixture is then centrifuged at 12000g for 1 min and the supernatant is carefully removed and the resin pellet is washed first with a high salt solution (3 gel volumes of gel solubilization buffer) and then with a low salt wash containing ethanol (Matrix wash buffer, Gibco). After centrifugation (12000g for 1min) the resin is air-dried (to remove the ethanol from the wash buffer) and 20ul of TE buffer (pH 8.0) are added and incubated 5 min at 50°C. After another centrifugation, the aqueous phase containing the DNA can be separated from the resin.

II.2.2.1.11 DNA Sequencing

At critical moment of our experiments, and after assembly of long stretches of DNA, we have confirmed the correct assembly of our constructs and the absence of mutations and frameshifts by automatic sequencing.

The following protocol is based of the user manual for cycle sequencing from the Perkin-Elmer corporation and is used in conjunction with an ABI PRISM 377 automatic sequencer running 36 wells gels.

The PCR reaction requires the following reagents:

Terminator ready reaction Mix (Perkin-Elmer): 8µl

Template: around 0.2µg/µl in a volume between 1.5µl and 2.5µl.

Specific primer: 5pmole

dH_2O: ad 20µl.

A reference sample, used as a positive control for the reaction, is added in every sequencing experiment. It is a pGEM vector used with a M13 (-21) primer.

The thermocycler program is the following:

The programs start when the lid of the cycler has reached 105°c

Step	Temp	Time	Action
			Rapid thermal ramp to 96°C
1	96°c	for 1 minute	Denaturation
2	96°c	for 10 sec	Denaturation
			Rapid thermal ramp to 50°C
3	50°c	for 5 sec	Annealing
			Rapid thermal ramp to 60°C
4	60°c	for 4 minutes	Elongation
5	-------	----------	The programs restart at step 2 for 25 cycles
			Rapid thermal ramp to 4°c
6	4°C	Until stop	

Upon completion of the sequencing reaction, the DNA is precipitated using conditions where unincorporated nucleotides do not co-precipitate. Unincorporated nucleotides need to be separated from the sequencing product, to prevent them to interfere with the sequence determination near the primer region.

In general, this can be achieved by Ethanol precipitation of the reaction. The reaction can be precipitated using 50µl of ethanol 95% and 2ul of 3M sodium Acetate, pH5.2.

The tube is the placed into a –80°c freezer for 30minutes and then centrifuged at 13000rpm for 20minutes. The supernatant was discarded as completely as possible. The pellet was then rinsed with 70% ethanol and centrifuged 20 minutes. The washing process has to be done twice. Alternatively, Centrisep spin columns (Princeton separations) can be used to purify the sequenced products.

Obtained pellets are resuspended using 3.5µl of a loading buffer composed of deionized formamide and 25mM EDTA (pH8.0) containing 50mg/ml blue dextran in a ratio 5:1 formamdide to EDTA/Blue dextran.
Before loading to the gel, the samples must be thoroughly vortexed to resuspend the pellet and denatured at 90°c for 2 minutes. The samples can be loaded immediately. Cooling of the samples on ice is NOT recommended.

Sequencing gels have been prepared according to the manufacturer's instructions.
The gels have to be prepared between two and six hours before loading. Preparation of the polyacrylamide gel is the most important variable that determines the number of bases that can be read when sequencing DNA. Polyacrylamide gels are formed by the copolymerization of acrylamide and bis-acrylamide (19:1 mix). The process begins when TEMED (N,N,N',N' Tetramethyethylenediamine) reacts with APS (ammonium persulfate) to yield a TEMED radical cation, a sulfate radical and a sulfate ion. The sulfate radical adds an unpaired electron to the acrylamide monomer, converting it to a free radical which then reacts with more monomer, causing polymer chain elongation. The polymer chains are randomly cross-linked with bis-acrylamide to form the gel matrix.

10X TBE stock solution, pH 8.3
Tris-base 108g (Sigma)
Borate 55g (Sigma)
$Na_2EDTA\ 2H_2O$ 8.3g (Sigma)

40% Acrylamide bis-acrylamide (19:1) stock solution
Acrylamide 38g (Sigma)
Bis-acrylamide 2g (Sigma)
H_20 ad 100ml
Acrylamide solution has to be de-ionized by stirring 10minutes with 10g of mixed-bed, ion-exchange resin and filtered on a 0.2µm filter.
The solution is very sensible to light and heat and must be conserved accordingly.

Gel solution:
Urea 18g (Sigma)
40% Acrylamide stock 5.5ml
dH_2O 25ml
Mixed bed ion exchange resin 0.5g (Bio-Rad)

The solution is stirred until all the urea crystals have dissolved then filtered on a 0.2µm cellulose nitrate filter and degassed for 2minutes.
5ml of 10xTBE solution is then added and volume is completed to 100ml with dH_2O.
Upon injection, 250µl of freshly prepared 10% APS (Bio-Rad) and 35µl of TEMED (ICN) are added to the gel solution and mixed. The solution should be casted immediately after adding the polymerizing reagents. Loading can be done 2hours after casting, when the gel is fully polymerized.

II.2.2.1.12 In vitro transcription of pSFV1 constructs

In vitro transcription of our pSFV1-cloned constructs has been performed to obtain RNA of our recombinant proteins. The obtained RNA will be used later for transfection of Eukaryotic cells.

First of all, the concentration of our DNA constructs should be precisely estimated by measuring their absorbance at 260nm. As a second step, all the vectors are digested using SpeI to linearize the constructs and prevent polymerization.

The rNTP, enzymes and Cap are provided by Promega. In a tube, add 100µl of rTTP 50mM, 100µl of rCTP 50mM, 100µl of rATP 50mM and 50µl of rGTP 50mM. The lyophilisated 5'cap is resuspended with 24,2ul of ddH_2O.

SP6 buffer 10x stock:	Reaction mix:
MgOAc 1M: 30µl (Sigma)	DNA (5 µg)
Spermidine HCl 100mM: 100µl (Sigma)	SP6 10x: 5µl
Hepes HCl 1M: 200µl (Sigma)	DTT 100mM : 2.5µl (Invitrogen)
H_2O: 170µl	Cap (50mM) : 1µl
	rNTP mix : 2µl
	ddH_2O : 35µl
	RNAsine (N2511) 40υ/µl : 1.5µl
	SP6 20υ/µl : 1.5µl

The reaction takes place at 37°C for 1 hour.
The obtained RNA has to be frozen at -80°C if not used immediately.

II.2.2.1.13 Transfection using electroporation for Eukaryotic cells

This technique will allow the transfection of RNA in BHK-21 cells to induce the synthesis of our proteins by eukaryotic cells. Due to the relative fragility of eukaryotic cells, it is extremely important to work quickly and on ice.

Sub-confluent BHK-21 cells are resuspended using Trypsine-EDTA. After a washing step in PBS without Ca^{++}/Mg^{++}, the pellet containing about 10^7 cells is resuspended in 10ml of PBS without Ca^{++}/Mg^{++}. Before electroporation, 800µl of BHK cells (about 8 10^5 Cells) is transferred to a pre-chilled cuvette containing 25µl of RNA solution.

The cuvette is then loaded in *Gene Pulser II* electroporation device (Bio-Rad) where two pulses of 850V, 25µF are applied. The duration of each of these pulses should be in a bracket of 0.36-0.40 milliseconds. The cells are then transferred into 75cm^3 tissue culture flaks containing 20ml of medium. The flasks are incubated 18h to 24h at 37°C then the cells are harvested using Trypsine-EDTA. The obtained pellet is sonicated 10 seconds to disrupt the cell walls and release the proteins. The supernatant is collected after a centrifugation at 500g for 10min.

II.2.2.2 Culture and induction of bacteria

II.2.2.2.1 Culture medium and agar plates

The bacterial cultures use Luria-Bertani growing medium and 50mg/L of ampicilin. All bacterial cultures are always incubated at 37°C with 150rpm agitation.

LB Broth (Luria-Bertani):
10 g Bacto-tryptone (Difco)
5 g Bacto-yeast extract (Difco)
5 g NaCl (Merck)
1 L water
Autoclaving is strongly recommended

For selection of resistant colonies, bacterial cultures are plated on Petri dishes containing agar. To prepare the agar plates, 7.5g of bacto agar (Difco) were added to 500ml of LB medium and autoclaved. The medium was then put into a 50°C waterbath until its temperature was stabilized and ampicilin (at a final concentration of 50mg/L) was added. The LB agar was then poured in sterile Petri dishes. The filled plated were let in a lamina flow until the LB was solidified.

II.2.2.2.2 Production of electro competent cells

Electroporation is a fast and efficient way to transform bacteria using plasmid DNA. In this process, E-Coli (DH5α) are prepared and washed to become electro-competent.

A fresh overnight culture was used to inoculate LB medium with a commercial stock of DH5α (the use of an "old" generation of electro competent DH5α decreases the efficiency of transformation). The inoculated culture is incubated at 37°c for about 5 hours until it has reached an optical density at 600nm of about 0.5 – 0.6. The culture is the centrifuged (5000rpm for 15min) at 4° and the supernatant is discarded. The bacterial pellet is resuspended in ice-cold sterile water and resuspended thoroughly. The centrifugation and washing steps are repeated twice. If the cells had to been frozen for a later use, the cells were resuspended in an equal volume of 10% Glycerol before freezing at –80°c.

II.2.2.2.3 Transformation using Electroporation for bacterial cells

Electroporation is a method of transformation that allows the introduction of foreign DNA into host cells (prokaryotic or eukaryotic) via the application of high-voltage electric pulses. The electric field induces pore formation in the cell wall and increases the permeability of the host cells to macromolecules, which allows for the uptake of DNA. Electroporation is a common method for the introduction of foreign DNA, such as plasmids, into *Escherichia coli.* (Lurquin, 1997) and can produce between 10^8 and 10^{10} transformed bacteria per µg of plasmid DNA.

The electroporation device used is a *Gene Pulser II* equipped with a *Pulse controller plus* and a *capacitance extender plus* (Bio-Rad). The conditions of the pulse were: 2500 Volts, 200 Ohms and 25 µFarad.

Ligated vector DNA at a concentration of 5pg to 10ng was added to 20ul of ice-cold competent cell. Sterile water was added up to 50ul and the mix was transferred into an electroporation cuvette.

The filled cuvette was inserted in the electroporation device and the pulse was applied.

An efficient transformation should show a pulse time of about 5 milliseconds. Lower values may results in poor efficiency due to high conductivity of the medium.

The cuvette was then filled with 1ml of freshly prepared SOC medium and put into a 37°c water bath for 30minutes, allowing the bacteria to recover in a medium containing high concentration of glucose. Two hundred milliliters of transformed bacteria are plated on an agar Petri dish containing selection antibiotics and incubated overnight at 37°C to amplify the individual clones.

SOB medium:
For 1liter:
20g Bacto-tryptone (Difco)
5g Bacto-yeast extract (Difco)
0.5g NaCl Merck)
900ml dH_2O
10ml KCl 250mM (Sigma)
Adjust to pH7.0 and autoclave

SOC medium:
For 50ml:
48.75ml SOB sterile medium
250ul $MgCl_2$ 2M (Sigma)
1ml Glucose 1M (ICN)

II.2.2.2.4 Glycerol Stock of transformed bacteria

As mentioned before, the use of bacteria for expressing our proteins offers us a great flexibility that allows for example to freeze a stock of our proteins for long term conservation. Bacterial clones in intermediate steps of constructs assembly and the final recombinant expression vectors were conserved as glycerol stocks at -80°C. These stocks made it easy to restart a new culture that will have the same properties as the original strain.

Conservation of our bacteria is simply done by mixing 850ul of bacterial culture (with an Optical Density at 600nm between 0.5 and 0.6) with 150ul of sterile glycerol. The vial is then vortexed and immediately frozen at –80°c or in a liquid nitrogen tank (for long term storage).
Cultures frozen in such a way have been shown to keep the vector intact for more that two years.

II.2.2.2.5 Induction of recombinant proteins using IPTG

For expression of recombinant proteins and polyepitopes, a set of vectors featuring a T7 promoter and a lac operator was used. These vectors are derived from Novagen's pET vectors, a plasmid for cytoplasmic production in *E Coli*, originally designed by Studier *et al* (Studier and Moffatt, 1986; Rosenberg *et al*, 1987; Studier *et al*, 1990). The expression is induced by providing a source of T7 RNA polymerase in the host cell. Plasmids are transfected into expression hosts (BL21DE3 cells) containing a chromosomal copy of the T7 RNA polymerase gene under LacUV5 control, and expression is induced by the addition of IPTG.

Before induction, a single colony from BL21DE3 transformed with our pET vectors is picked and used for inoculating 50ml of LB containing 100mg/L of Ampicilin. Alternatively, 10-20µl of a glycerol stock can be used for inoculating the LB.

The Erlenmeyer flask is incubater at 37°C with shaking until the OD at 600nm reaches 0.4-0.6. IPTG 0.5mM is added to the flask and induction continues for 3-4 hours. The cells can then be harvested by centrifugating at 5000g for 5min at 4°C. The cell can then be lysed to recover the periplasmic fraction containing the recombinant proteins.

This protocol was generic and showed a poor efficiency with some of our proteins. Its was therefore improved by several modifications concerning the time of induction or the concentration of IPTG. This part is further developed in the results section.

II.2.3 Protein purification and characterization

II.2.3.1 Extraction of soluble protein from bacterial cells

The vector's manufacturer was recommending a lysis using Tris-HCl and EDTA followed by a second step in 5mM $MgSO_4$. As preliminary tests have shown that a proportion of the protein was still contained in the cytosolic fraction of the cell, we decided to use a different lysis technique to recover a maximum quantity of our proteins. Moreover, the EDTA contained in this protocol would have perturbated the planned purification using HPLC Nickel columns (stripping agent). A lysis protocol using physical disruption and no EDTA was chosen instead.

At the end of the induction, the culture is centrufugated at 5000g for 5min. The supernatant is discarded and the pellet is frozen at -80°C for 30min then reheated at 37°C for 5minutes. The thawed pellet is then ultrasonicated for 10minutes in presence of 5ml of PBS. This process is repeated 3 times. At the end of the third cycle, the pellet is resuspended in a solution containing 6M guanidine HCl pH 7.8, 20mM $NaPO_4$ and 500mM NaCl and centrifugated at 3000g for 15min. The supernatant obtained contain up to 2ug/ul of total protein.

To concentrate the obtained supernatant, the samples can be passed on Microcon 10kd spin-columns and washed with PBS.

II.2.3.2 Purification of histidine-tagged recombinant proteins by metal chelate chromatography

Soluble recombinant proteins from bacterial lysates were HPLC purified under native conditions on imobilised metal affinity chromatography columns (IMAC). Recombinant proteins presenting a hexa-histidine tag are retained on a chromatographic matrix presenting bivalent metal ions at pH 7.4. The metal ions most often used are nickel (Ni^{2+}) copper (Cu^{2+}) and zinc (Zn^{2+}).

The strong interaction of penta- or hexa-histidine motifs with bivalent metal ions allows to retain proteins with a histidine tag on an IMAC matrix while non his-tagged proteins are removed from the column by a washing step. Elution is generally obtained by increasing the imidazole concentration in the mobile phase. Alternatively a decrease in pH can be applied to elute the bound proteins. Imidazole competes with the histidine motifs for the binding sites (chelated bivalent metal ions) on the matrix, leading thus to the desorption of bound proteins. At low imidazole concentrations histidine containing proteins are eluted, whereas histidine-tagged recombinant proteins display a stronger interaction with the matrix and require generally higher imidazole concentrations for elution to occur, allowing thus for the preparation of a relatively pure recombinant protein in one purification step. If the optimal imidazole concentrations for washing and elution are determined the separation can be realized as a two step procedure.

Optimal his-tag mediated binding may be reduced for specific proteins. The folding properties of a given recombinant protein may reduce the accessibility of the purification tag for the matrix bound ions and thus reduce the yield of the purification. In these cases purifications under denaturing conditions, using buffers containing urea or guanidinium hydrochloride, may render the IMAC purification possible but require a subsequent refolding step to restaure the initial protein conformation.

We compared native and denaturing conditions for the purification of several of the recombinant proteins used in this study. Since the yields were comparable we prefered the the non-denaturating buffer system.

Chromatographic separations were done with a HPLC system, the AKTA explorer 10S station (AP Biotech) coupled with a fraction collector, the FRAC950 (AP Biotech). This equipment consists of a two pump gradient former for a precise control of elution conditions and a variable wavelength UV detector for the monitoring of the protein content by absorbance measurement at 280nm and 230nm. Eluate fractions of 200ul were collected in ELISA plates for convenient detection of relevant protein fractions after chromatographic separation.

The chromatographic columns were 1ml HiTrap chelating columns (AP Biotech, Nr: 17-0408-01) with a maximal binding capacity of 12 mg his-tagged protein / ml matrix. The matrix (Chelating Sepharose High Performance) consists of highly cross-linked agarose beads to which iminodiacetic acid has been coupled by stable ether bounds via a seven-atom spacer arm.

The charging of the chelating matrix with Ni++ was achieved by injecting 0,5ml of a 0,1M $NiSO_4$ solution. Non chelated nickel ions were removed from the column during a washing step with 5 column volumes of water before the column was equilibrated at a flow of 1,0 ml/min. in the starting buffer, 100% buffer A (20mM phosphate buffer, pH 7,4, 500mM NaCl). To avoid cross-contaminations we reserved one column per protein sample, following the instructions of the manufacturer for matrix regeneration.

A representative chromatogram (Fig 25) shows the different steps of the purification of recombinant proteins with a hexa-histidine tag.

The soluble fraction of the bacterial lysate was diluted in buffer A prior to injection on the column. The protein sample was loaded on the column by repeated injections of 1ml aliquots, using a 1 ml sample injection loop. Non retained proteins were collected in a "flow-through"fraction (fraction Fr3). Upon completion of the sample injection the column was washed with 10 column volumes of buffer A. The wash was collected in fraction Fr4.

Elution of matrix bound proteins was achieved using a gradient of buffer B (20mM phospate buffer, pH 7,4 ,500mM NaCl, 500mM imidazole) in buffer A. This gradient (green curve, %B shown on right y axis) increases the percentage of buffer B from 0 to 30%B in 3 column volumes, from 30 to 97%B to in 4 column volumes, from 97 to 98% in 9 column volumes. A final increase to 100%B cleans the column and stops fractionation prior to re-equilibration in starting buffer. After onset of gradient the baseline absorbance at 280nm increases due to the increased concentration of imidazole in the mobile phase.

The eluate between 2% and 100% B buffer was collected in 200ul fractions in 96 well plates. Proteins were dectected by UV monitoring at 280 nm (blue curve, left Y axis). After overnight incubation the fractions were transferred in a fresh 96 well plate and the 96 well plate used for fraction collection served to test the eluate fractions for reactivity with specific moAbs in ELISA.

The results of the ELISA assay (red dotted line) are overlayed as an activity histogram on the chromatogram.

Peak A, which is eluted at low imidazole concentration, contains bacterial proteins which are weakly bound the Ni++ column, the his-tagged recombinant protein is eluted in peak B.

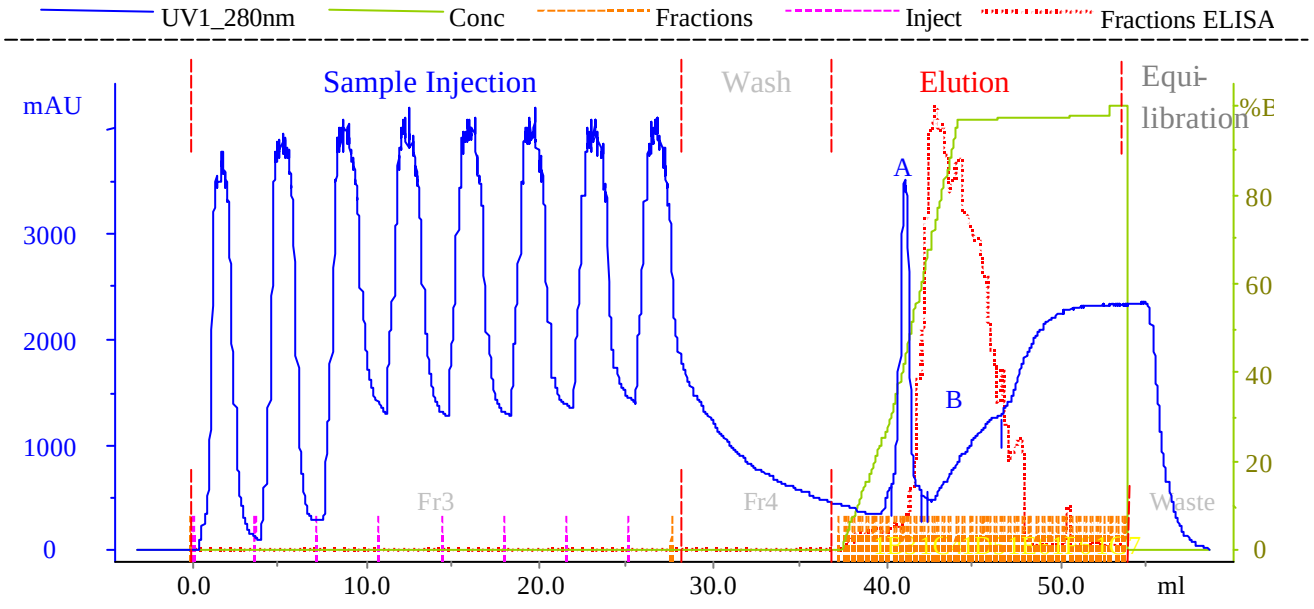

Fig 25: Chromatographic purification of his-tagged recombinant protein

Fractions containing antibody reactive his-tagged proteins were subsequently pooled. The pooled proteins were desalted and concentrated using centrifugal filter devices (microcon or centricon, Millipore) with appropriate size exclusion membranes (10 kD or 30kD) to retain the proteins of interest. The retained proteins were thus transferred in PBS 1x buffer, quantified and used for further tests and immunizations.

The elution of histidine-tagged proteins by applying a linear imidazole gradient (brown dotted line) under native conditions (20mM Phosphate, pH7.6, 500mM NaCl) has been monitored at 280nm (blue line). Eluate fractions (200?l) have been tested in ELISA with an anti-his moAb and the signals (OD_{405nm}) of the fractions are represented as an overlayed histogram (Fig 25 and Fig 26).

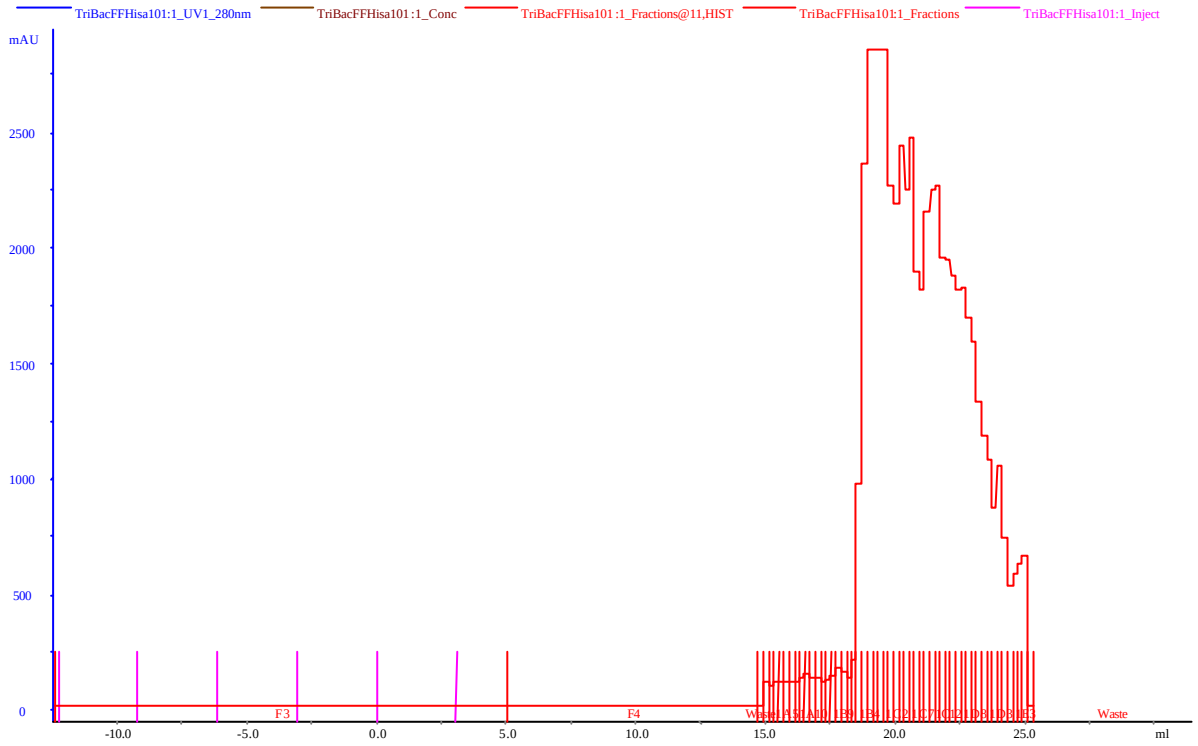

Fig 26: IMAC purification of his-tagged poly-mimotopes from soluble bacterial protein fraction
The red line shows the protein elution curve

IMAC Buffers :

	MW				
Na2HPO4 – 2 H2O	177.99	1.42 g (*1.13g)	7.1 g (*5.66g)		* if Na2HPO4 anhydrous
NaH2PO4-H2O	137.99	1.11 g	5.55 g		
NaCl	58.44	23.38 g	116.9 g		
H2O		90ml	450ml		
		100ml	500ml		

pH is then adjusted to 7

II.2.3.3 Protein quantification using Bradford reaction (biorad, cat n°500-0116**)**

The determination of protein concentration is extremely important in the course of our experiments for the reliability and reproducibility to SDS page, ELISA and other tests, such as immunization with recombinant protein. The test we are using is derived from the Bradford reaction, but with a quicker and more stable coloration.

A standard curve is prepared using 5 dilutions of a reference protein (Bovine serum albumin) of known concentration. The absorbance at 750nm of these dilutions is linear from 0 until 1.5 ug/ul.

10 ul of every reference and test samples are pipetted into a well of a flat-bottom 96 wells plates. 20ul of the reagent A of the kit are then added to the samples and mixed. After shaking thoroughly, 180ul of the reagent B are added to the plates and mixed with the pipette. After 15 minutes, the results can be read at 750nm in an ELISA reader. The values will remain stable for at least 1hour.

The values obtained by this method can be easily analyzed by plotting the reference values into an excel graph, and using the “trend line” function to obtain the formula of the line. This function can then be used to calculate precisely the concentration of the unknown samples.

II.2.3.4 Protein separation using electrophoresis (SDS-PAGE)

To assess the efficiency of our expression system, we analysed bacterial lysate of expression clones using denaturating protein gels, SDS-PAGE (SDS PolyAcrylamide Gel Elecrophoresis).

One-dimensional electrophoresis using discontinuous gels is a high resolution method used to separate mixtures of proteins to investigate subunit composition.

Our protocol is based on the standard Laemmli method (Laemmli, 1970), used for discontinuous gel electrophoresis under denaturing conditions (using 0.1%SDS). Dithiotreitol (DTT) can be added in the samples before loading to reduce the disulfide bonds to minimize protein aggregation.

Casting of the gels:

The gel is composed of two parts: A separating gel, where the actual size separation will take place, is casted first. The acrylamid concentration can vary between 6% and 15%, defining the gel porosity and thus the separation range. The stacking gel is casted on top of the separating gel. This stacking gel will concentrate the sample before entering the separating gel.

The gel material is home made and consists of two 9cm x 9cm glass squares assembled on 1mm thick spacers and held together using metal clamps. Agarose is poured on the side and bottom of the gel casing to prevent leakages.

Separation gel composition:

For 10ml of separating gel mix

Density	6%	8%	10%	12%	15%
Optimal range	150-60kD	70-45kD	55-30kD	35-15kD	25-5kD
H_20	5.3ml	4.6ml	4.0ml	3.3ml	2.3ml
30% acrylamide mix	2.0ml	2.7ml	3.3ml	4.0ml	5.0ml
1.5M Tris (pH 8.8)	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
10% SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% APS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
TEMED	0.008ml	0.006ml	0.004ml	0.004ml	0.004ml

Remark: The TEMED is a polymerization catalysor that works only at basic pH. The pH of the solutions is critical to ensure correct polymerization.

After casting, the gel is covered with a layer of isopropanol to prevent oxidation. The gel is let to polymerize for 30minutes. When the gel is polymerized, the isopropanol is removed and the stacking gel is casted.

For 4ml of stacking gel

H_20	2.7ml
30% acrylamide mix	0.67ml
1.5M Tris (pH 8.8)	0.5ml
10% SDS	0.04ml
10% APS	0.04ml
TEMED	0.004ml

Right after casting, the comb is inserted into the stacking gel. The gel is the allowed to polymerize 30-60 minutes and the transferred to the electrophoresis tank (X-Cell 2, Novex). The tank is immediately filled with the electrophoresis buffer to prevent the gel from drying.

Electrophoresis buffer (final concentration):
25mM Tris (Sigma)
250mM glycine (pH8.3) (Sigma)
0.1% SDS (Merck)

Prior to sample loading, the wells are rinsed with a syringe and a long needle to remove the non-polymerized acrylamide.
Before loading, the samples are mixed with 1 volume of loading buffer containing SDS:

50mM Tris-HCl (pH 6.8) (Sigma)
2% SDS (Merck)
0.1% bromophenol blue (Serva)
10% glycerol (ICN biomedicals)

100mM (final concentration) of dithiotreitol (DTT) can eventually be added in the mix.
The samples are then boiled for about 3minutes to unfold the proteins.
After loading of the sample, the electrophoresis is connected to a power supply (E835, Consort) at 100V, 80mA until the bromophenol blue reaches the bottom of the gel.

The gel can then be used for coloration using Coomassie brilliant blue or for western blot analysis.

<u>Products used:</u>
Tris (Sigma)
Glycine (Sigma)
SDS: Sodium Dodecyl sulfate (Sigma)
Tris-HCl (Sigma)
Bromophenol blue (Serva)
Glycerol (ICN biomedicals)
APS: Ammonium persulfate (Sigma)
30% Acrylamide mix: Consists of a mix of 29% of acrylamide (Sigma) and 1% of N,N'-methylenebisacrylamide (Sigma) in ratio weight/volume in deionized water.
TEMED: N, N, N',N'-tetramethylethylenediamine (Bio-Rad)

II.2.3.5 Reversible protein detection kit (Sigma, cat ref R-PROB)

This kit is an analog to the Ponceau S reaction for reversible staining of proteins on membranes and presents the great advantage of a reduced background on PVDF membranes after destaining. The protocol has been slightly modified. After the blotting process, the membrane is washed 5 minutes and incubated with the protein detection kit solution for about 15 minutes. The membrane is then washed with ddH_20 until the desired contrast is achieved. The membrane can then be scanned or photographed. Finally, the coloration is removed using EDTA 50mM prior to immunostaining.

II.2.3.6 Coomassie brilliant blue coloration

Coomassie staining is one of the simplest non-radioactive methods to visualize proteins in gels. To color a gel using Coomassie brilliant blue, 125 mL of methanol is combined with 25 mL of glacial acetic acid and 100 mL of dI water to make 250 mL of destaining solution. 0.1g of Coomassie Brilliant Blue R-250 is dissolved in 50 mL of the destain solution to make 50 mL of Coomassie staining solution. The gel can then be immerged in the Coomassie staining solution on a rocking platform shaker for 1 hour to overnight. Increasing the time can improve the detection of possible faint protein bands. After coloration, the Coomassie stain is discarded and the gel is washed in destain solution until the background in the gel is clear. The destaining usually takes 1 hour or longer and the solution can be replaced with fresh solutions several times as necessary.

II.2.3.7 Western Blotting

Western blotting (or immunoblotting) will allow us to identify proteins separated by SDS-page using monoclonal antibodies. This experiment consist to transfer elelectrophorically the proteins from the gel to a PVDF membrane (Bio-Rad) using a semi-dry transfer apparatus. The membrane-bound proteins are then probed with a selection of antibodies.

In the semi-dry blotting, the gel is placed in contact with the PVDF membrane and compressed between buffer-saturated Whatmann paper in contact with the electrodes, with the following organization:

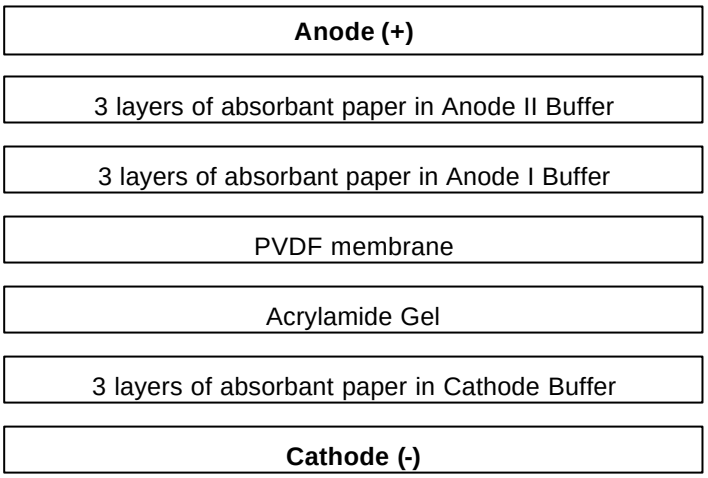

Fig 27: Structure of a Western blot "sandwich"

The electrodes are then connected to the generator and a current of 8V, 100mA is applied to the assembly for 1hour.

Cathode buffer:	Anode Buffer I:	Anode Buffer II:
25mM Tris-Base 40mM 6-aminohexanoic acid 20% methanol	30mM Tris-Base 20% Methanol	300mM Tris-Base 20% Methanol

At the end of the transfer, the membrane is colored with Ponceau S or Reversible protein detection kit (Sigma).

The membrane can then be prepared for immunostaining using mono- or polyclonal antibodies. First, all remaining binding sites have to be blocked with PBS, 0.1% Tween20 and 5% powdermilk (Gloria, Nestlé) for 1hour with a gentle agitation. At the end of the blocking, the membrane has to be carefully washed using PBS 0.1%Tween20 (3 times 2minutes). The membrane is then incubated 1 hour with the diluted antibody and subsequently washed as indicated in the previous step. The antibody-antigen complex is then incubated with a Goat Anti-mouse IgG labeled with alkaline phosphatase (Southern biotechnology associates) during 1 hour, with a gentle agitation. After the washing step, the substrate (5-Bromo-4-Chloro-3-Indolyl phosphate/Nitro blue tetrazolium tablets, Sigma) is added to the

membrane to monitor the activity. The reaction is usually quick and can be stopped after 5 to 15 minutes depending on the signal intensity and background staining.

II.2.3.8 Proteinase inhibitor

To prevent our cell lysate containing our proteins to be degradated by proteases, we have added to our lysates a preservation mix made of several proteinase inhibitor:

Leupeptin: 10µg/ml (Sigma)
PMSF (Phenylmethylsulfonyl fluoride): 10mM (Roche)
Apoprotein: 10µg/ml (Sigma)
Iodoacetamide: 108mg/ml (ICN)

II.2.4 Animal experiments

II.2.4.1 Animal anesthetics

During the course of our animal experiments, Mice anesthesia was sometimes needed for critical manipulations requiring easy access to the mouse's body during, for example, blood collection or ear-tagging. The described injectable anesthetic mixture paralyses the mouse and leaves it for 10 to 15 min in stable position for facilitated handling.

For 4ml of anesthetic mixture, mix:
0,5ml of Imalgene 1000
0,5ml of Rompun 1:10 in PBS
1ml of Atropinum sulfuricum 1:10 in PBS
2ml of PBS

The mixture should be prepared freshly before injection. A volume of 100 ul/10mg of body weight is injected into the peritoneal cavity using 1ml syringes. The mice fall asleep 2-5 minutes after injection.

The anesthetic mixture reagents can only be ordered by veterinary prescription:

Imalgene 1000 (ketamine 100ng/ml) (Merial)
Rompun 2% (Xalyzine 20mg/ml) (Bayer)
Atropinum sulfuricum 1% (WDT)

II.2.4.2 Immunization and boostings

The most critical and final step of our experiment is the animal experiment. During this long process, our constructs will be injected in the mice that will generate an immune response towards our constructs.

Production of good antisera depends in large part upon the quality, purity and amount of available antigen as well as on the specificity and sensitivity of the assay. Immunization of inbred mice allows the study of precisely defined antibody specificity along with high reproducibility of the results due to the high similarity between individuals. The adjuvant used is the Freund's adjuvant (Freund *et al*, 1937).

Every construct will be injected to a group of five Balb-c mice ($H2^d$). A week prior to immunization, every mouse is bleed to collect a sample of "naïve" reference serum. The day of immunization, one volume of a solution of 0,5ug/ul of each construct is mixed with one volume of complete Freund's adjuvant.
The solution is mixed in a glass tube using a glass syringe until a stable emulsion is obtained.
Two hundred µl of emulsion are then injected in the peritoneal cavity of each mouse.
Boosting injections are done in the same way, but using incomplete Freund's adjuvant instead.

After the primary immunization, naïve B cell of the mice are stimulated to differentiate into antibody-secreting plasma cells. For most soluble protein antigens, specific antibodies begin to appear 5 to 7 days after the animal is injected. The antibody concentration continues to rise and peaks around day 12 after which it decreases. In addition to differentiating into antibody-forming cells, the stimulated B cells proliferate to form a large population of memory B cells, which quickly become activated after the booster injection is administered. Thus, the lag period before the appearance of the specific antibody is much shorter after a booster injection than that observed for the initial immunization. In addition, a significant higher titer of specific antibodies is achieved and maintained for a longer period of time. The peak of antibody production occurs 7 to 14days after boosting. Finally, the average affinity and degree of specificity of the antibody population for the antigen increase with repeated immunizations (Klinman *et al*, 1976)

After the first injection, the next boost will be scheduled 4 weeks later, followed by a blood sampling the next week. A second and third boost followed every 15 days.

In our case, it meant the following schedule:

Day 1	**Day 7**	**Day 35**	**Day 42**	**Day 50**	**Day 60**	**Day 66**	**Day77**
Pre-Bleeding	Immunization	Boost1	Bleeding1	Boost2	Bleeding2	Boost3	Bleeding3

To allow immunization with a homogeneous batch of proteins, every immunization and boosts were done with the same protein pool, obtained by the pooling of the bests samples (batches) of proteins produced and purified.

II.2.4.3 Blood sampling in the retro-orbital plexus

To monitor the humoral immune reaction generated by the injection of our recombinant proteins, regular blood sampling (bleeding) was necessary.

The easiest way to get a sufficient (100 to 200ul) amount of serum is the blood sampling in the retro-orbital plexus.

Prior to sampling, the mice are anesteziated (following the protocol described in unit II.2.4.1). Then the experimentator gently pull the skin of the mouse's skull from the opposite side of the head and the eye will pop out. A glass capillary can then be inserted between the eye bulb and the retro-orbital plexus. Gently pushing the tip of the glass capillary on the array of veins behind the eye generates a quick and abundant bleeding that can easily be stopped by pushing the eye back into place. However, this manipulation requires calm and dexterity not to wound the mouse.

For the last blood sampling, a higher quantity of blood can be collected by cardiac punction using a syringe, after killing the mice by cervical dislocation.

After collection, the blood is incubated 30min at 37°C and centrifuged 30 min at 3000rpm. The serum is then aliquoted and frozen at -20°C.

Part III

Results

III. Results

In the first part of the Results Section, we will present the results obtained of study of the outbreak that occured 1996. In the second part of the Results Section, the development, construction and expression of chimeric proteins as the basis of a novel vaccination approach will be described.

III.1 Secondary immune response in adults with low levels of antibodies

III.1.1 Description of the phenomenon

Secondary immune responses (SIR) against measles have been observed in fully immunized populations (Gustafson *et al*, 1987). With vaccination rates on the increase, it becomes increasingly urgent to understand the role of individuals without symptoms, such as those with inapparent SIR, in measles virus transmission (Pedersen *et al*., 1989).

The secondary immune response of parents from the children with measles was investigated during a measles outbreak that happened in Luxembourg between March and July 1996. All children had clinical measles according to the Centers for Disease Control case definition (CDC, 1990) and developed specific IgG. 46 children were tested positive for IgM (Fig 28) and showed a significant increase of specific IgG titers when paired sera were available (Fig 29).

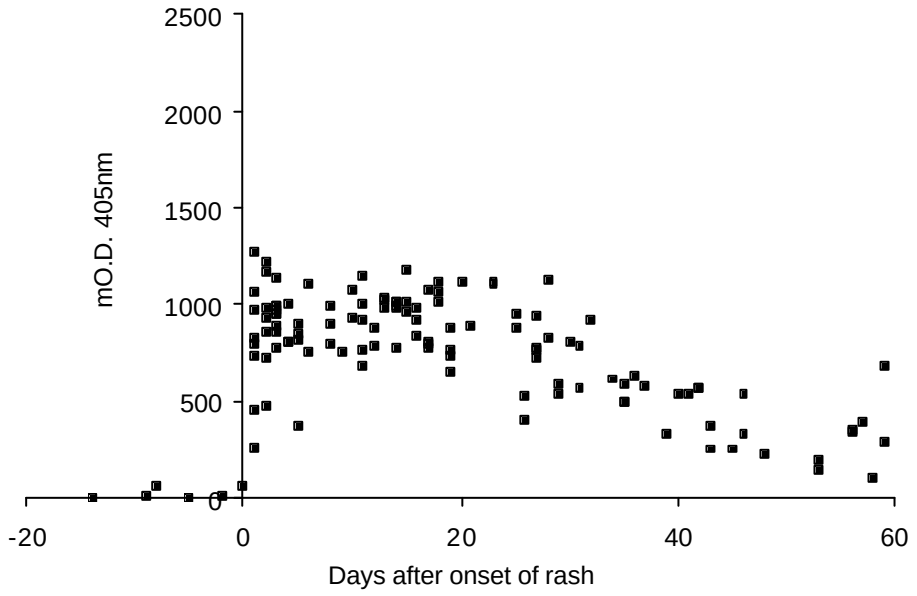

Fig 28: Evolution of the patients IgM titer after MV infection

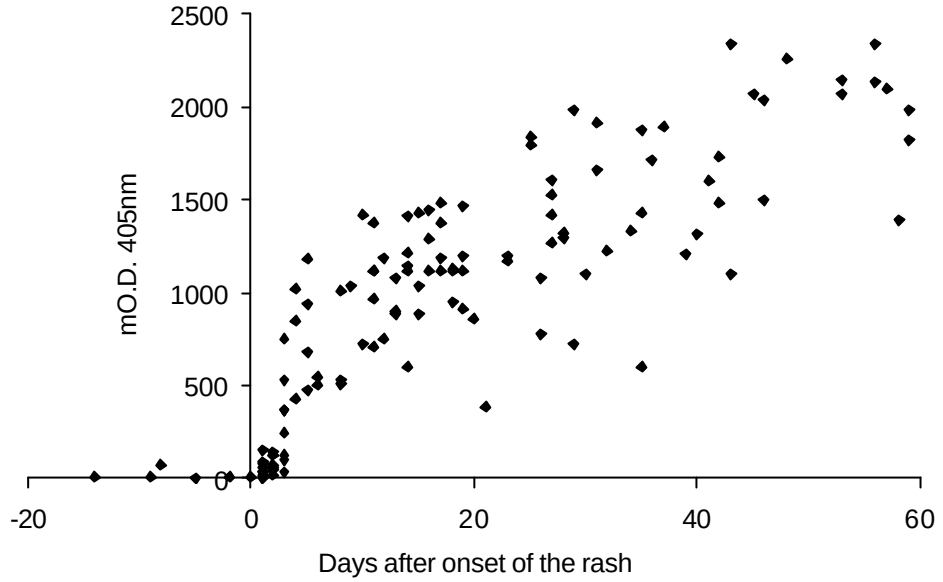

Fig 29: Evolution of the patients IgG titers after MV infection

We have investigated whether immune parents would respond to the re-exposure to a measles-infected person. 45 parents (37 mothers, 7 fathers, 1 grand-mother), for which 1 to 7 pre-exposure sera were available, were considered having been exposed while taking care of their children with measles. Some parents were exposed to up to 4 children with measles. The average age of the parents was 37.2 years (range 26.3-50.5 and one with 67.5 years). All parents reported that they were not vaccinated and that they have had measles before entering school. Pre-exposure sera, obtained during routine venupuncture for unrelated reasons between August 1990 and March 1996, were available from the serum bank of the Laboratoire National de Santé. The interval from the last pre-exposure sera to the exposure was 2-66 months. The post-exposure interval was between 2-12 weeks. None of the parents reported any measles-related symptoms during the time of the study.

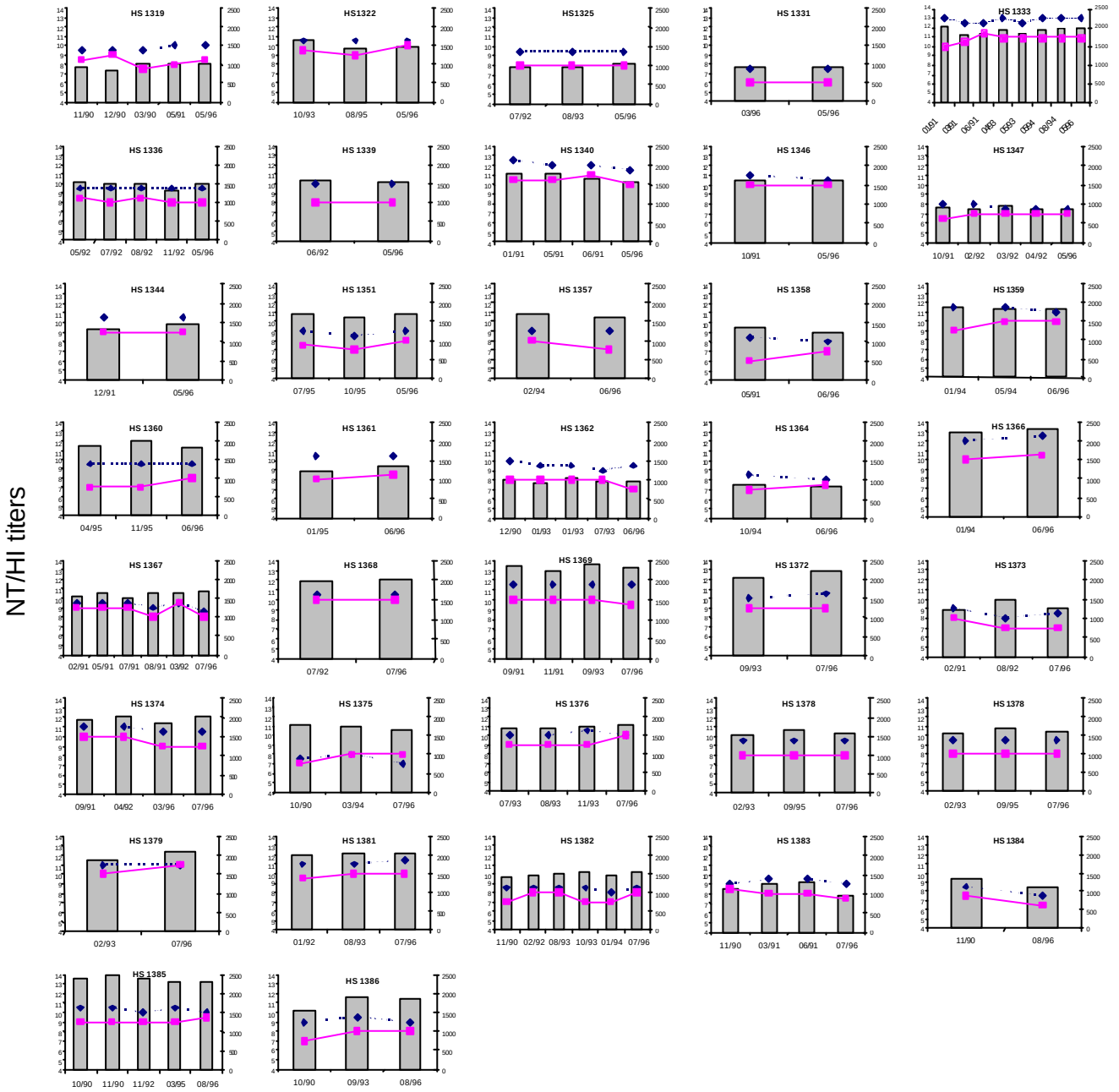

Fig 30: Specific IgG (grey bars), NT (■) and HI (◆) titers of all available pre-exposure sera of the parents, in chronological order. In each graph, the last point to the right (May to August 1996) is serum drawn immediately after the outbreak

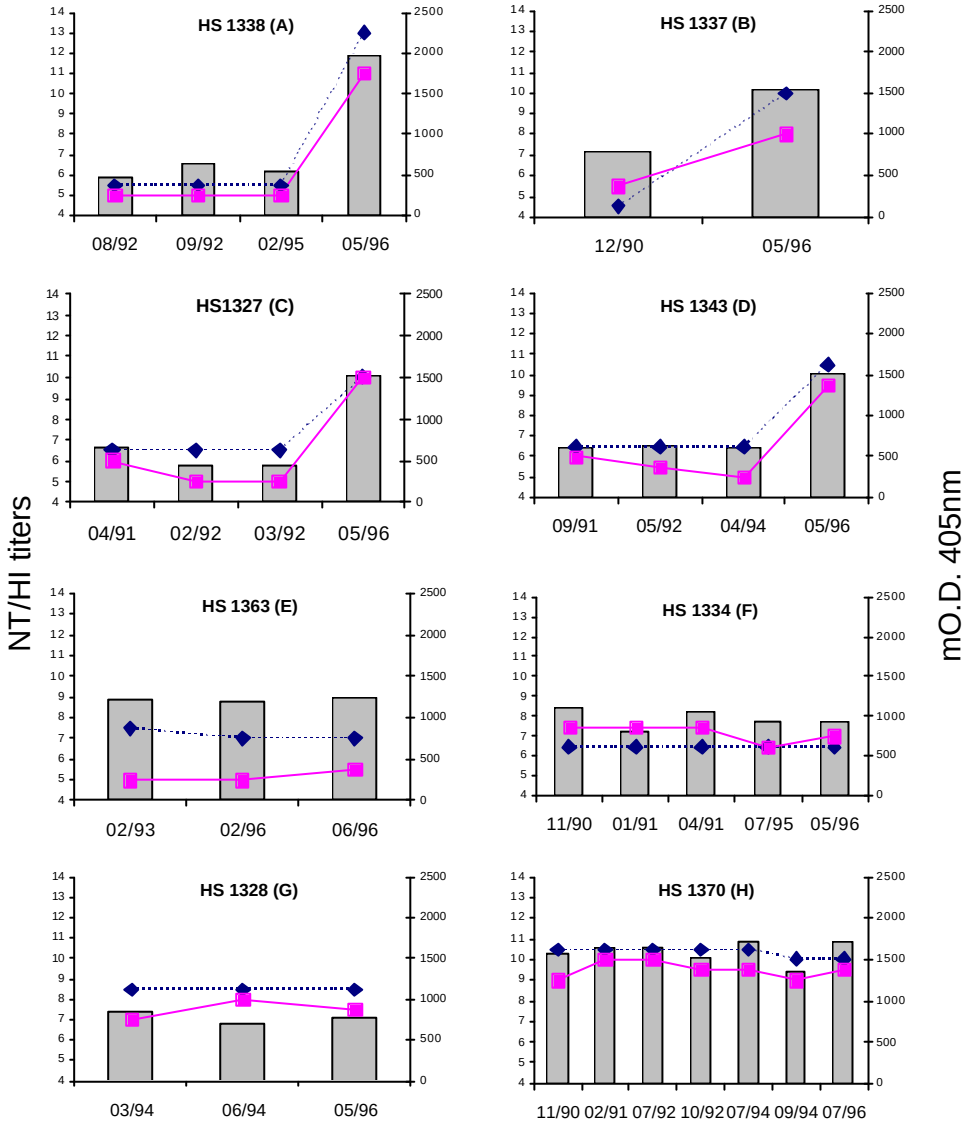

Fig 31: Specific IgG (grey bars), NT (■) and HI (◆) titers of parents with high variation of titers. In each graph, the last point to the right (May to August 1996) is serum drawn immediately after the outbreak.

Fig 30 and Fig 31 show that, prior to exposure, sera of all the parents were clearly MV-IgG positive (ΔA>400 mO.D.), according to the manufacturer's specifications of the ELISA (cut-off for positivity: "corrected" ΔA>200 mO.D.; cf. Materials and Methods). In the sera of Fig 30, no significant changes in titers (<2-fold dilution) were observed during the 66 months before exposure. Also after measles exposure, most of the parents showed no significant changes in antibody titers as tested by either one of the 3 assays, however (Fig 31) four parents HS1327, HS1337, HS1338 and HS1343 (refered to as C, B, A, D respectively) with lower IgG titers showed a significant and concomitant increase of NT (>4-fold increase, $p<0.005$ by paired t-test of pre- and post-exposure sera) and HI titers (>8-fold; $p<0.006$). If the serum titer of the parents did not change during during measles re-exposure, the values obtained with the pre-exposure sera should be similar to the values of the post-exposure sera. None of the other parents showed a significant increase in post-exposure titers above a 2-fold dilution ($p>0.1$). Although IgG levels of some of the other parents shown in Fig 30 and 31 had antibody titers only slightly higher (as parents 1363, 1334 and 1328) these did not develop SIR by any of the parameters measured here.

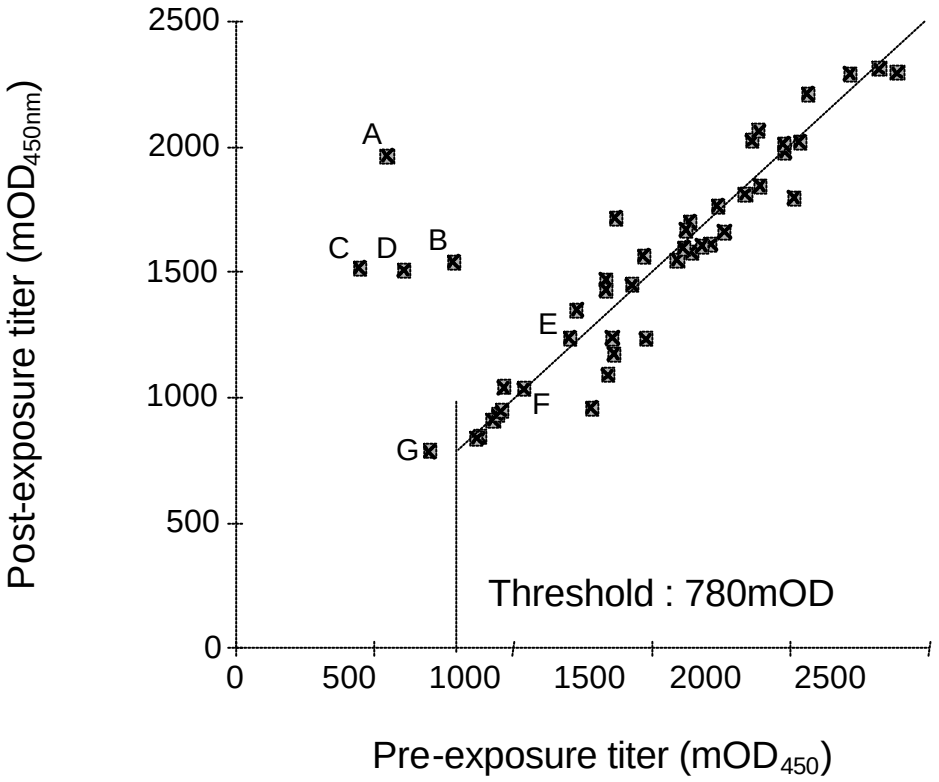

Fig 32: Comparison of last pre-exposure and post-exposure IgG-titers of parents (n=45) of measles children.

In Fig 32**,** specific IgG reactivity of the last pre- and post-exposure IgG levels of all parents are shown. All but 4 parents exhibit no significant difference between pre- and post-exposure IgG levels. Above a pre-exposure level of 780 mO.D., post-exposure antibody levels were <150 mO.D. higher than pre-exposure titers (pre- vs. post-exposure MV-IgG above 780 mO.D. pre-exposure absorbance: $p>0.5$, by paired t-test)**,** except for one parent with considerable fluctuations in pre-

exposure IgG levels (parent H or HS1370 : ΔA=300 mO.D.) not seen in his NT or HI titers, or in any other parent. Below 780 mO.D., measles antibodies of 4 out of 5 parents were boosted by 750-1400 mO.D. (pre- vs. post-exposure MV-IgG below 780 mO.D. pre-exposure absorbance: $p<0.02$, by paired t-test). Pre-exposure titers of parents with and without boosted antibodies were also significantly different ($p< 10^{-4}$).

This shows that some parents developed SIR when re-exposed to measles. This observation was also confirmed by similar data obtained in HI and NT tests.

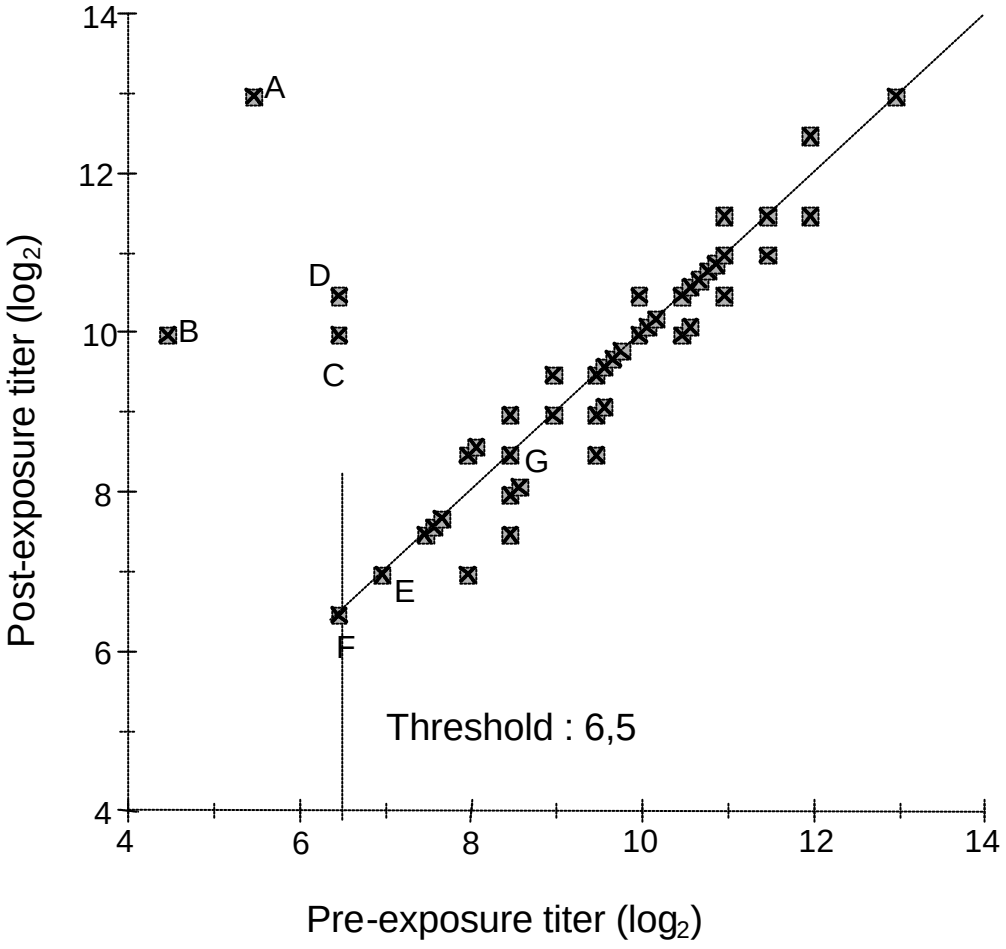

Fig 33: Comparison of last pre-exposure and post-exposure HI-titers of parents (n=45) of measles children

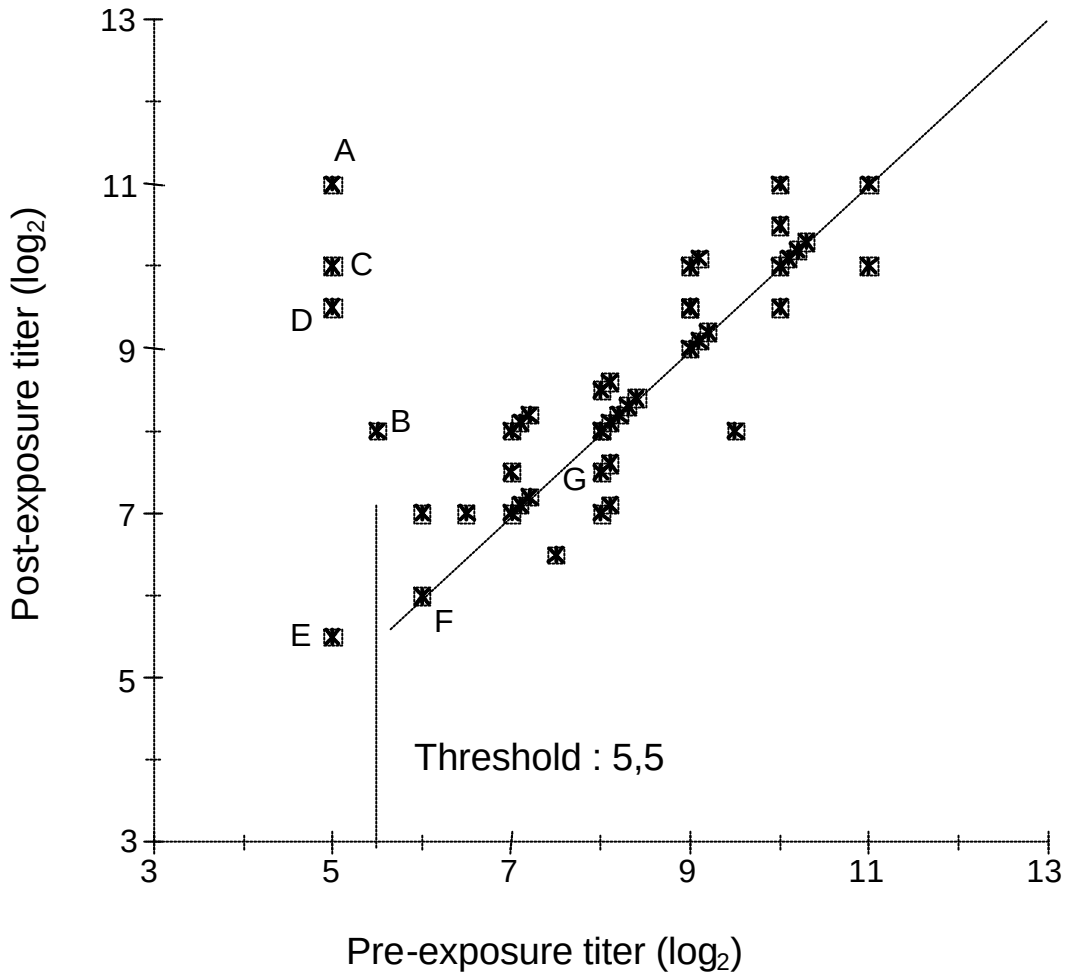

Fig 34: Comparison of last pre-exposure and post-exposure NT-titers of parents (n=45) of measles children

Fig 33 and Fig 34 show the pre-exposure HI and NT plotted against the corresponding post-exposure titer. The individuals (designated by A, B, C, and D in Fig 32, 33 and 34 (or 1338, 1337, 1327,1343 in Fig 31) were also lowest for their pre-exposure NT and HI titers. Above defined pre-exposure titers ($1:2^{5.5}$ dilutions for NT; $1:2^{6.5}$ for HI), no significant booster effect was observed (pre- vs. post-exposure titers: $p>0.1$ by paired t-test). Pre-exposure NT and HI titers of SIR-parents were significantly lower than pre-exposure titers of parents without SIR (NT: $p<10^{-12}$; HI: $p<10^{-3}$ by non-paired t-test). The comparison of pre-exposure NT and HI titers of parents with and without SIR also shows that, while individual G was below the IgG threshold (780 mO.D.), she was well above the HI and NT threshold. Conversely, parents E and F are below the threshold for NT and HI respectively, but not for IgG. Parents E, F, and G did not undergo SIR, suggesting that one parameter may not be sufficient to predict SIR.

IgM levels of the parents were also tested using IgM Enzygnost kits (Fig 35) and were negative (<200mO.D.) in all the parents, including the SIR parents. During primary measles responses, IgM

levels of ΔA>200 mO.D. are considered positive, while ΔA<100 are negative according to the manufacturer's instructions. Such values are not necesseraly valid for SIR.

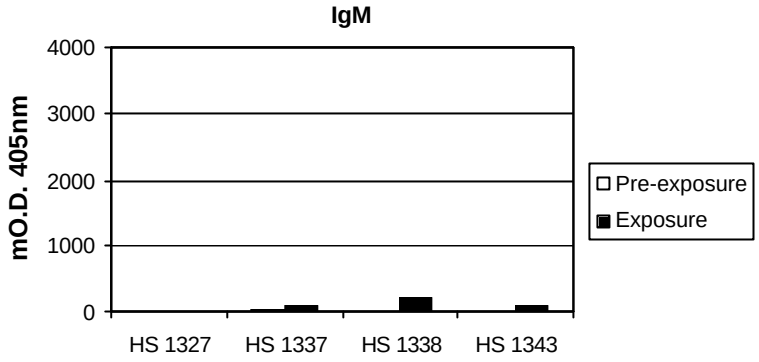

Fig 35: IgM levels of the 4 SIR parents

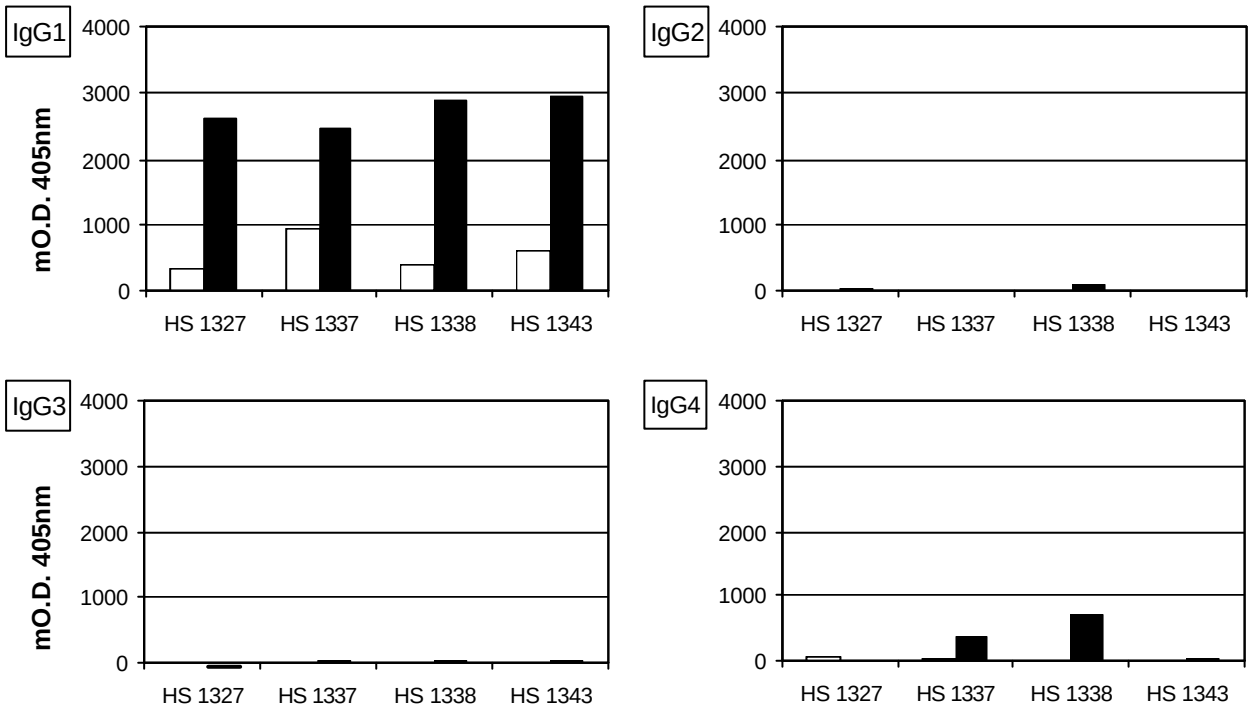

Fig 36: IgG subclasses of the 4 SIR parents

Fig 36 shows that among the IgG subclasses, mainly IgG1 was boosted during the SIR. Two parents which were IgG4 negative, became positive for this subclass after exposure (HS1337 and HS1338), but this subclass showed elevated background values (500 mO.D.). IgG2 and IgG3 were essentially negative, before and after exposure.

The results suggest that the SIR seems to be an "all or none response" since parents with IgG, NT or HI levels just above the defined threshold do not develop SIR. This threshold become further evident when the pre-exposure values of the 3 tests are plotted in a single graph (Fig 37).

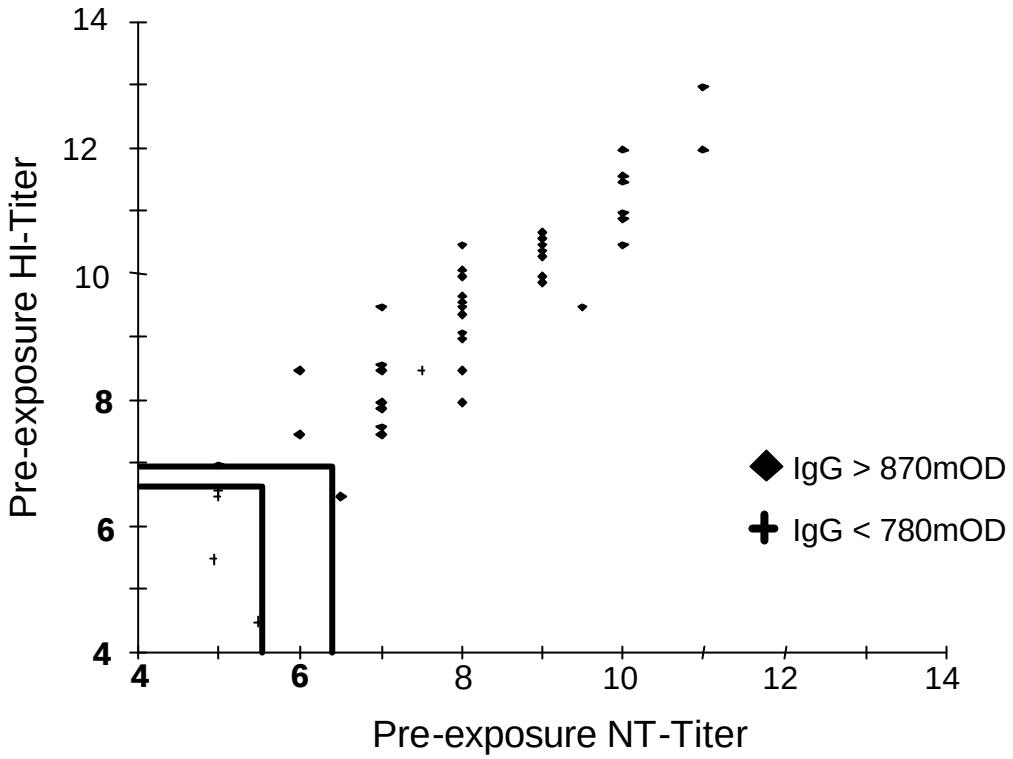

Fig 37: Combined HI, NT and IgG pre-exposure values

Fig 37 shows the pre-exposure levels of IgG, NT and HI with the corresponding thresholds were observed in the previous figures. The brackets between the highest values of individuals with SIR and the lowest values of non-SIR individuals delimit a “gray zone” in which the SIR-susceptibility is not defined. Using these serological characteristics, it may be possible to identify individuals susceptible to SIR when exposed to a measles patient using the combined thresholds (Table 3). Fig 37 also shows that all parents were positive by in vitro neutralization (>1:32) and by hemagglutination inhibition (>1:22), i.e. at least for the lowest dilutions tested.

	Minimal Threshold	Maximal Threshold
Enzygnost (IgG)	780 mO.D.$_{405nm}$	870 mO.D.$_{405nm}$
NT	$1:2^{5.5}$	$1:2^{6.5}$
HI	$1:2^{6.5}$	$1:2^{7}$

Table 3: Combined threshold describing SIR-susceptibility.
Minimal and maximal values define a grey zone where SIR susceptibility is undetermined

In Fig 37, the thresholds are shown as lines which define an area in which the SIR-susceptibles can be found. In this specific group, the proportion of SIR-susceptibles reaches 9% (4/45). All the SIR parents were women.

By transposing this frame of threshold to other groups or populations, it may be possible to estimate the proportion of SIR-susceptibles in these populations. In the next section, we will

estimates the proportion of SIR-susceptibles persons in a group of children that recently got measles (Early Convalescent), in a group of adults that got measles during their childhood (Late convalescents) and in groups of school children vaccinated once or twice against measles.

III.1.2 Estimation of susceptibility to SIR

III.1.2.1 Description of the cohorts

In the following, we apply the above serological characteristics to different populations in order to estimate the proportion of SIR-susceptibles in these populations. The proportion of SIR-susceptible persons were estimated in a group of children that recently recovered from measles (Early convalescent), in a group of adults that had measles during their childhood (Late convalescents) and in a group of school children who were vaccinated either once or twice against measles.

Fig 38 shows the relative distribution of HI and NT titers and specific IgG for late convalescent donors and high school children stratified according to their vaccination status. Sera from early convalescent measles patients, drawn at a time when the antibody response was fully developed in most patients (i.e. 14-59 days), are shown for comparison.

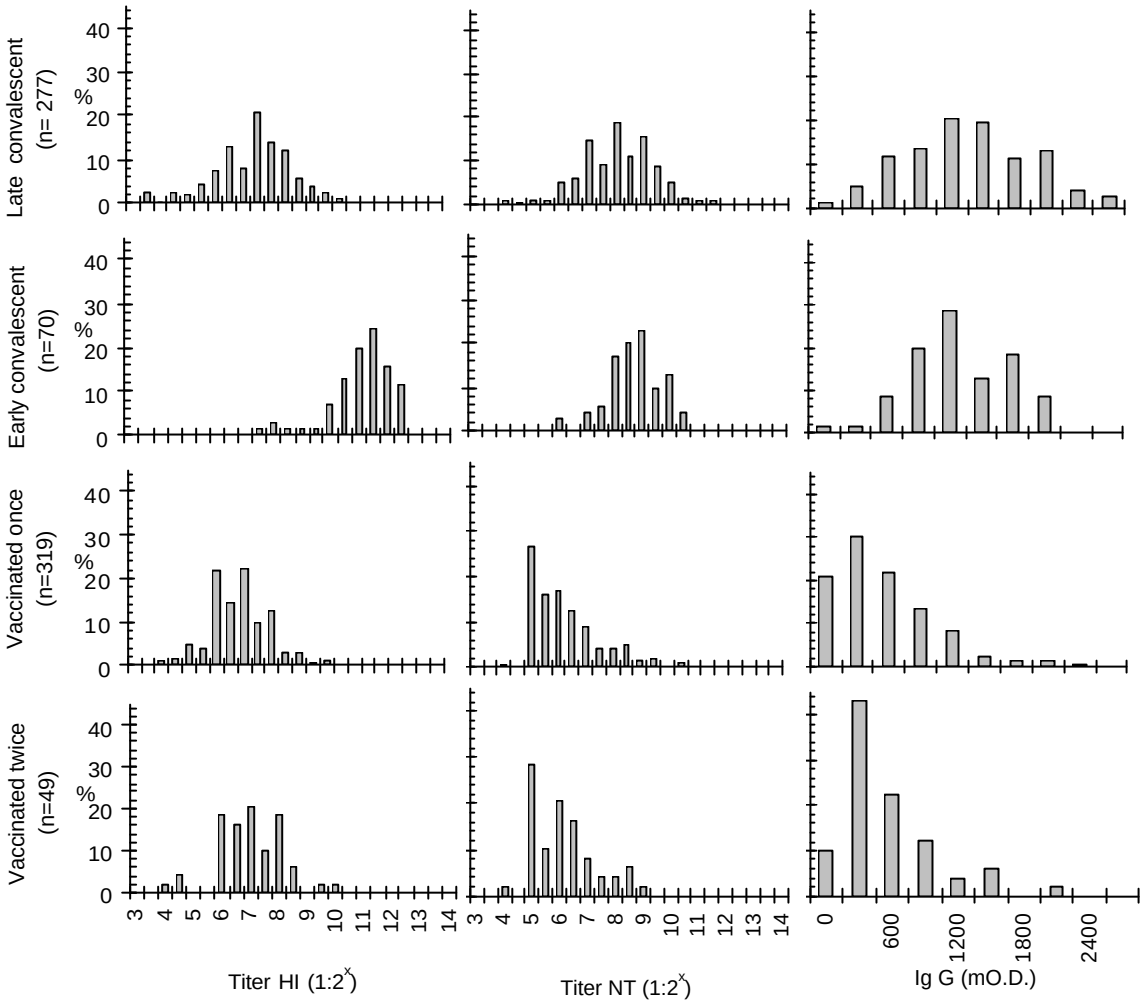

Fig 38: Comparison of the different cohorts

	HI		NT		IgG	
Cohorts	Median	St Dev	Median	St Dev	Median	St Dev
Late convalescent	7.5	1.39	8.0	1.26	1501	599
Early convalescents	11.5	1.09	8.75	0.98	1424	464
Vaccinated once	7.0	1.12	6.0	1.21	587	463
Vaccinated Twice	7.0	1.16	6.0	1.24	591	449

Table 4: Median and standard deviation of the cohorts from Fig 38

Assays	Cohorts	Cohorts		
		early convalesc.	late convalesc.	vaccinated (1x)
HI	early convalesc.	-----	-----	-----
	late convalesc.	$<10^{-60}$	-----	-----
	vaccinated 1X)	$<10^{-100}$	$<10^{-6}$	-----
	vaccinated (2x)	$<10^{-38}$	n.s.	n.s.
NT	early convalesc.	-----	-----	-----
	late convalesc.	$<10^{-4}$	-----	-----
	vaccinated 1X)	$<10^{-42}$	$<10^{-57}$	-----
	vaccinated (2x)	$<10^{-24}$	$<10^{-21}$	n.s.
MV-IgG	early convalesc.	-----	-----	-----
	late convalesc.	n.s.	-----	-----
	vaccinated 1X)	$<10^{-22}$	$<10^{-59}$	-----
	vaccinated (2x)	$<10^{-14}$	$<10^{-17}$	n.s.

Table 5: Levels of significance (P by unpaired Student T-Test) of HI, NT and IgG between Early and Late convalescent donors and vaccinated school children.
n.s. not-significant

Early convalescents have significantly higher MV titers (by HI, NT and specific IgG) than late convalescents or vaccinees (see Table 4 and 5). This observation indicates that the immunity obtained after measles infection is waning. The difference is much more important in HI than in NT and IgG. Late convalescent persons had significantly higher NT and HI titers than individuals vaccinated once indicating that natural measles infection provide better and longer protection that the immunity provided by vaccination. After a second vaccination, HI titers but not NT titers became similar to those of late convalescents. No statistical difference was found between children vaccinated once or twice although some individuals with the lower HI titers seem to have converted to higher HI titers. This indicates that a second vaccination do not icrease the immunity level but gives a second chance to the individuals that did not respond to the first vaccination. Within the cohorts of once- or twice-vaccinated, no serological differences between boys and girls or between different schools were detectable by Student t-test. Also, no significant difference in titers was detected between the different measles vaccines used (Rimparix- and Pluserix) (data not shown).

III.1.2.2 Susceptibility to SIR

Fig 39 to 42 show the HI and NT titers combined with levels of specific IgG of convalescent and vaccinated donors in relation to the described thresholds of Table 3. Different estimates of the frequency of SIR-susceptibles are obtained whether sera within the undefined “gray zone” were included or not. The more conservative (i.e. lower) estimate is obtained when individuals with values within the undefined region are considered SIR resistant.

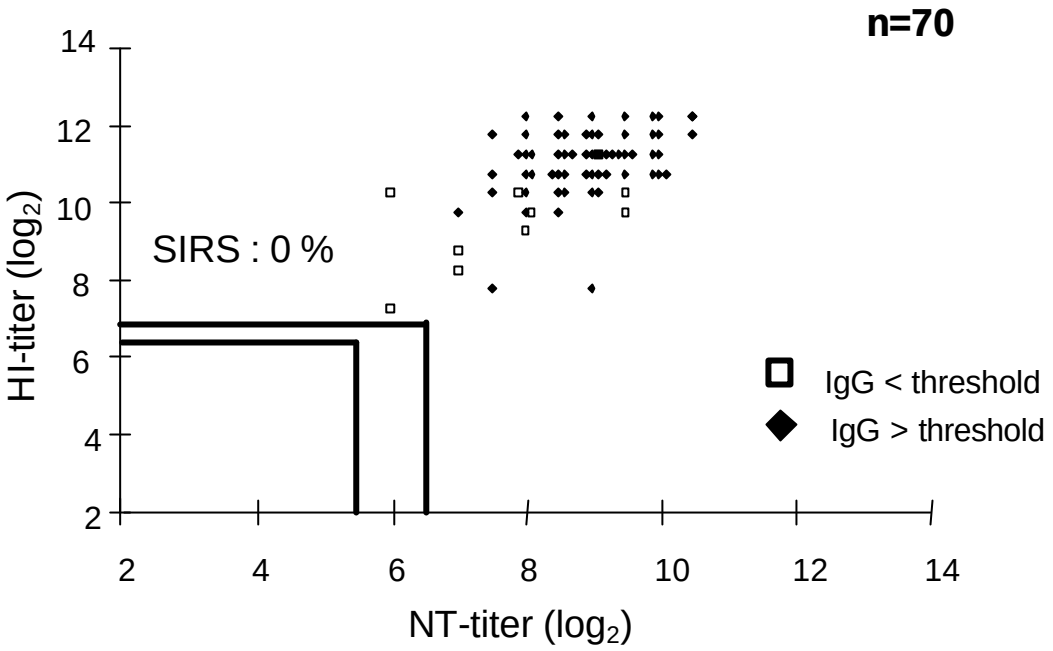

Fig 39: Titers of early convalescents.

As expected, none of the early convalescents fulfills the criteria of SIR susceptibility (SIRS). Their recent, full scale Measles infection generated high levels of circulating antibodies that will protect them against disease as well as reinfections

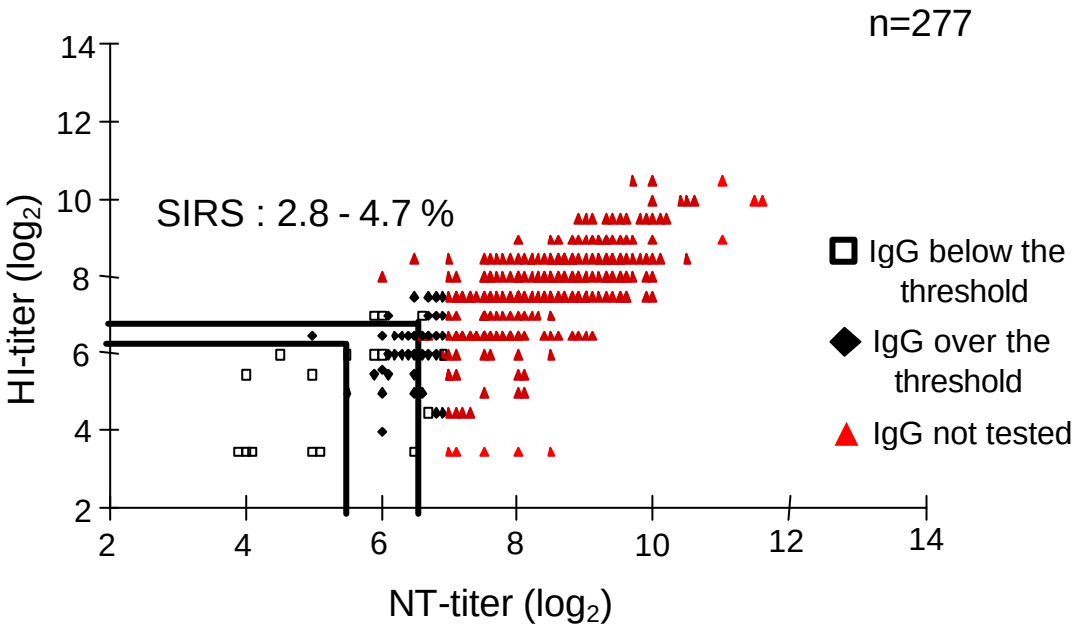

Fig 40: Titers of late convalescents

Among the late convalescents only 3.2-3.9 % (1 male, 8 females) seems to be SIR-susceptibles which is lower than the 9% of SIR-susceptibles found in the parents of measles children (not significant).

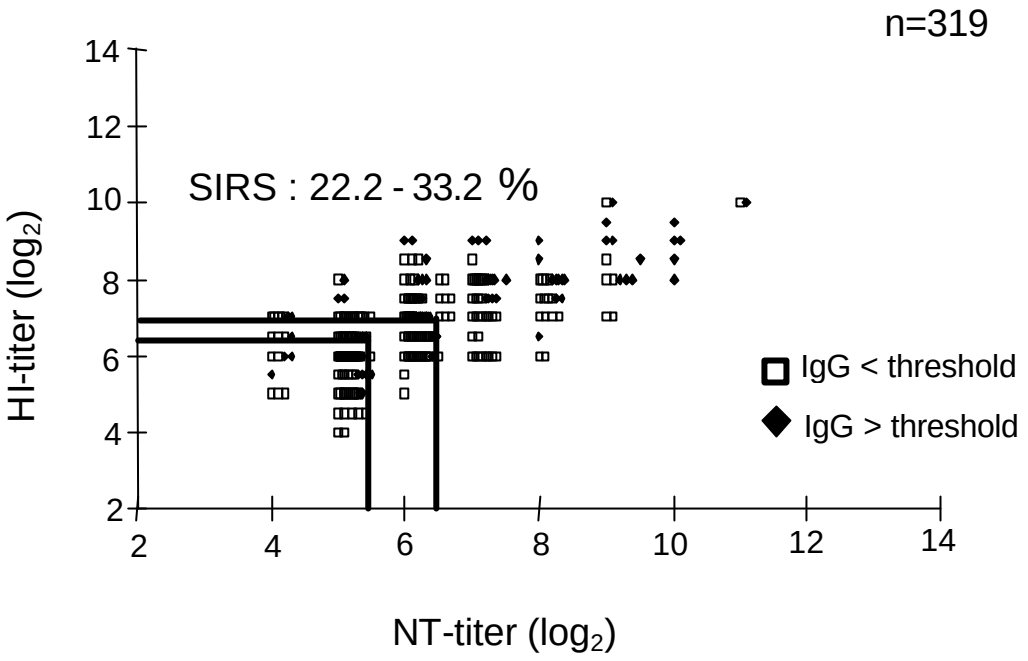

Fig 41: Titers of children vaccinated once

In Fig 41, it can be seen that among the vaccinated school children the frequency of SIR susceptible vary from 22.2% to 33.2%. This is 7-8 times higher that for the late convalescent group, suggestion that vaccination may generate a higher proportion of SIR-susceptibles.

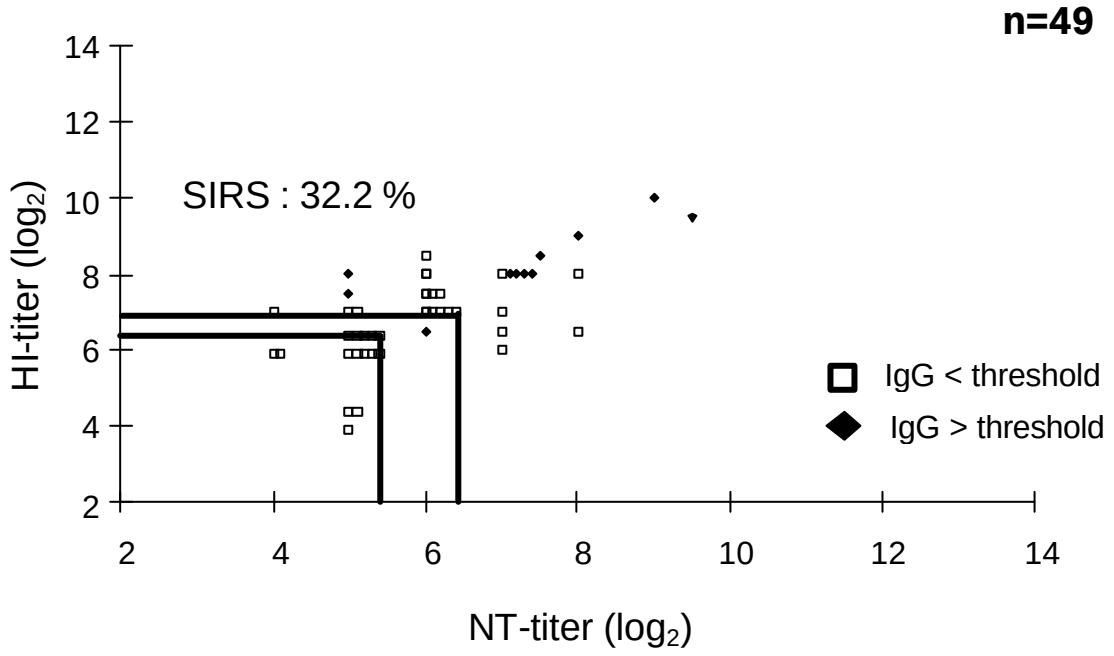

Fig 42: Titers of children vaccinated twice

In the group of children vaccinated twice, the proportion of SIRS is 32.2%, a value equals to the group vaccinated once suggesting that a second vaccination do not seems to reduce the proportion of SIR-susceptibles.

In all of the above figures, the estimated frequencies of secondary immune response would be higher if only two parameters were considered although a reasonable approximation would be obtained in a cohort of late convalescents, but not in vaccinees, by combining NT titers and Enzygnost data. Another point is that the SIR-susceptible late convalescents tended to be younger in comparison to the average age of this cohort (33.6 versus 43.5 years; P=0.07). All late convalescents were born before 1973 (five before 1965) and 63.5% were women.

Among the vaccinated children, no difference in sex distribution or the vaccines used was found between SIR-susceptibles and resistant subpopulations (male/female for SIR-susceptibles and non-SIR susceptibles: 0.21 and 0.29). In the SIR-susceptible and resistant groups of vaccinees a similar percentage (8.6 and 7.8%) was vaccinated before the age of 15 months. With a single exception all SIR-susceptibles were vaccinated before the third birthday, whereas in the resistant (non-SIR) group, 17.2% were vaccinated after this date. The latter may have had contact with wild-type MV before being vaccinated which would explain their higher titers. Alternatively, vaccination at a later age may induce higher titers.

III.1.3 Duration of the booster effect induced by the secondary immune response

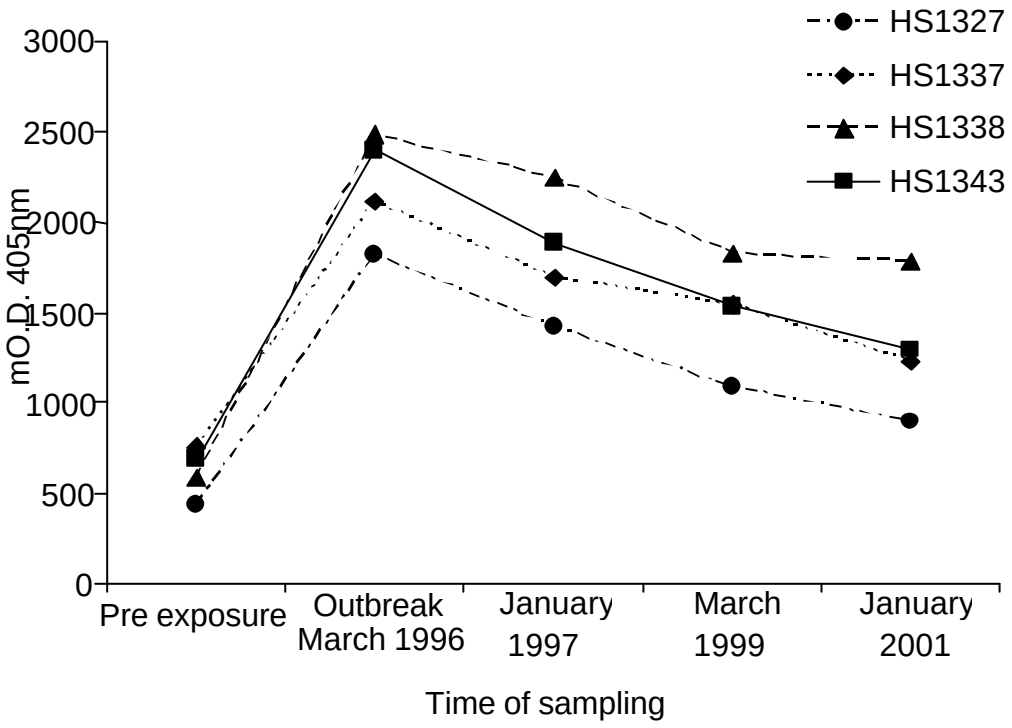

Fig 43: Duration of booster effect

Fig 43 shows the evolution of the titer of the four SIR parents. The graph shows that after the boost generated by the measles contact, their immunity is rapidly decreasing. The booster effect seems then to be relatively transient. Parents HS1343, HS1327 and HS1337 have lost around 30% of their boost 6 month after the outbreak although it takes a bit longer for parent HS1338 to reach this

level. The 4 parents have lost on average 42% of their boosted immunity 42 months after the outbreak. Table 6 shows the relative decrease of the immunity of the 4 parents.

Parent :	Outbreak Mars 1996	January 1997	March 1999	January 2001
HS1327	100%	71%	48%	34%
HS1337	100%	69%	58%	35%
HS1338	100%	88%	66%	63%
HS1343	100%	70%	50%	35%
Average	100%	75%	55%	42%

Table 6: Decrease of the titer of the 4 parents

III.2 The vaccine strategies

The previous section has shown that the neutralizing humoral response is much weaker in vaccinees than in late convalescents. It is furthermore known that vaccine-induced immunity wanes much faster than immunity due to natural infection. In some studies, a considerable proportion of vaccinated children have lost their immunity within less than a decade (Ratnam *et al*, 1996; Ramsay *et al*, 1994; Guris *et al*, 1996; Aaby *et al*, 1999). In other studies, it is estimated that 50% of the vaccinees have lost their immunity within 25 years after vaccination and that 80% of vaccinees will eventually become unprotected (Mossong *et al*, 1999). The previous Section shows also that, in contrast to late convalescents, a considerable proportion of vaccinees may not be protected against infection although they are protected against disease. Thus the current vaccine may protect less efficiently than previously thought and considerable efforts are made to develop improved vaccination strategies. Here we propose to develop antigens that are based on selected T and B cell epitopes of the most immunodominant proteins as a complement to the live attenuated vaccine.

Another group of susceptibles for whom there is currently no vaccine available are young infants before the age of 9-15 month. These children are partially protected by maternal antibodies. Thus the vaccine should be resistant to preexisting anti-(whole) measles virus antibodies. Furthermore, to avoid priming of T cells for atypical measles, such a vaccine should not contain measles-specific sequences that could serve as T cell epitopes.

Vaccines for the above applications require a vastly different design. The following sections describe the development and the construction of these antigens.

III.2.1 Design and development of the antigen : Polyepitopes based on mimotopes

III.2.1.1 Selection of epitopes and building of the constructs

This strategy was meant to explore the potential of a conformational epitope of the measles virus fusion protein that was mimicked by a peptide mimotope. The litterature has shown that the fusion protein is the target of efficient neutralizing antibodies (Malvoisin and Wild, 1990) and that the specific mimotope selected for this study generates *in vivo* protective antibodies in a laboratory animal model (Stewart *et al.*, 1995). Here, we generated chimeric proteins containing the mimotope sequence and selected T cell epitopes. The conformation of the mimotope is critical for the induction of neutralizing antibodies. The number and position of the sequences flanking the mimotope are likely to be critical for the proper cross-reactive neutralizing immunogenicity. Since the influence of the flanking sequences cannot be predicted, we have chosen a permutational approach to build series of chimeric polyepitopes in which the mimotope is embedded in different microenvironments.

The selected epitopes included:

The **T cell epitope F256-270** (LLGILESRGIKARIT) is an epitope in the mouse described by Muller *et al* (1995). This peptide was selected from 108 pentadecapeptides of the measles fusion protein and is part of a cluster of strong $H2^d$ restricted T cell epitopes.

A **MV-F derived mimotope** (NIIRTKKQ), described by Steward *et al* (1995) as a B cell epitope. This mimotope has the sequence of a peptide selected from a solid-phase random combinatorial octamer peptide library (synthesized on a polystyrene-polyethylene resin using the Fmoc chemistry) using a monoclonal antibody against the MV-F protein, F7-21. This peptide shows some structural similarity with F protein of MV and CDV. Steward *et al* (1995) showed that this peptide induced a good antibody response and was significantly protective against a neuroadapted MV or CDV after immunization together with a T cell epitope. Althrough this mimotope was referred to in the publications of Steward *et al* (1995) as M2, in our constructs we designated it as M for simplicity.

The **TT 830-844 "universal" has been described as a promiscuous human T cell epitope** (QYIKANSKFIGITEL), (Kumar *et al*, 1992). A so-called promiscuous T cell epitope is able to be presented by a wide range of MHC-II haplotypes and ensures T cell activation in the majority of the human population. It is derived from the tetanus toxoid (TT). High antibody titers were obtained when immunization is done in the presence of this "universal" T cell epitopes (Kumar *et al*, 1992; Hathaway *et al*, 1995; El Kasmi *et al*, 1999).

The following constructs were assembled by genetic fusion of the above epitope sequences (F refers to the TCE F256-F270, T to the tt830-844 fragment of the tetanus toxoïd and M to the mimotope):

Sequence	Molecular weight
2xT-8xM-2xF	16730
2xF-8xM-2xF	16560
2xF-8xM-2xT	16730
4xM	4500
8xM	9000
2xF-8xM-2xF-8xM-2xF-8xM-2xT	42290
2xT-8xM-2xF-2xF-8xM-2xT	33460
2xT-8xM-2xF-2xF-8xM-2xF	33290
2xF-8xM-2xT-2xF-8xM-2xT	33460
2xF-8xM-2xT-2xF-8xM-2xF	33290
2xF-8xM-2xF-2xT-8xM-2xF	33290
2xF-8xM-2xF-2xF-8xM-2xT	33290
2xF-8xM-2xF-2xF-8xM-2xF	33120
4xT-8xM-4xF	24460
4xF-8xM-4xT	24460
4xF-8xM-4xF	24120
8xT	15800
8xT-4xT-4xF	31260
8xF	15120
8xF-4xF-4xT	30580
16xM	18000

Table 7: List of constructs using minimal epitopes

III.2.1.2 Expression and testing of the constructs

Oligonucleotides coding for the above epitopes were synthesized as single-stranded DNA (by Eurogentec, Liège, Belgium) and then annealed into double-stranded DNA. The synthetic DNA fragments encoding the epitopes were flanked by restriction sites, designed to allow oriented cloning, a Sal I site at the 5'end and a XhoI site at the 3'end. Such fragments served as base-units for the preparation of polyepitopic constructs.

After assembly of the epitopes following the procedures described in the Materials and Methods, the constructs in Table 7 were removed from pUC 18 and inserted into the pSFV1 expression vector using the Bam H1 restriction enzyme. The constructs were then transcribed into RNA *in vitro*. This was followed by transfection of the RNA-constructs into BHK-21 cells. The expression of

recombinant protein in transfected cells was analyzed by flow cytometry (FACS). Cell lysates were obtained and tested in ELISA using polyclonal sera generated with peptides, corresponding to the M, F256 and TT830 sequences, or the F7-21 monoclonal antibody (courtesy of Prof. Osterhaus, Netherlands) which was used to select the mimotope M2 (Steward *et al*, 1995). None of these detection methods revealed the presence of recombinant protein in the transfected cells.

Although the constructs could not be detected by the above antibodies, transfected cell lysates were tested by T cell proliferation assays to detect the presence of the TCE of the recombinant protein.T cell lines specific for the TCE F256-270 and the TT830-844 were generated and were used to compare the potential of the constructs to stimulate T cells.

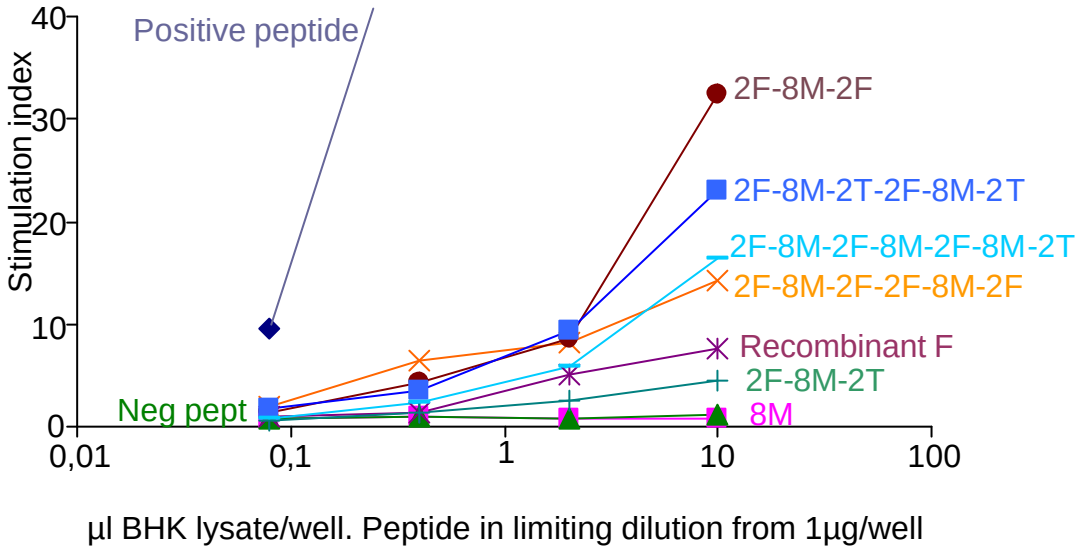

Fig 44: Stimulation of F256 specific T cell line by polyepitopes

Fig 44 shows that the cell lysates induced a significant IL2 production of the F256 specific cell line. The “2F-8M-2F” constructs induced the strongest proliferation. This could be due to its 4 copies of the F256 epitope. The analoguous but longuer “2F-8M-2F-2F-8M-2F” construct gives a somewhat weaker T cell stimulation perhaps as a result of lower expression. The “2F-8M-2T-2F-8M-2T” construct gives a better T cell stimulation than 2F-8M-2F-2F-8M-2F although the latter incorporates twice as much F256 epitopes. Properties of the constructs during the synthesis (RNA structure and stability, protein half-life) or position and flanking sequences of the epitopes may explain that result. The “8M” constructs, as well as the negative peptide, did not induce any proliferation, confirming the specificity of the stimulation index.

The T cell assay confirms that the constructs were processed and probably presented by MHC class II restriction elements. It also confirms that the constructs were produced in the cells, but surprisingly the mimotope was not recognized by the monoclonal antibody F7-21.

Since the recombinant constructs were not detected by mab F7-21 neither in ELISA nor by flow cytometry, synthetic peptides corresponding to the TCE and BCE constructs alone or in combination were injected into mice to generate specific sera. All M units in the recombinant constructs were flanked by the two amino acids leucine and aspartic acid, which are left over of the SalI–XhoI restriction sites required for the assembly of the polyepitope constructs. To investigate a possible influence of the spacer amino acids on the epitope conformation, two M-dimers differing only by the spacing amino acids between the mimotopes were synthesized as peptides.

Thus, the two peptide dimers were:
Mimotope – **Leu – Asp** - Mimotope (spacer as in the recombinant protein)
Mimotope - **Gly – Gly – Gly** – Mimotope (neutral spacer).

These peptides were used for immunizing mice. The sera obtained with the synthetic peptide containing the glycine spacer (gly-gly-gly) reacted strongly with the corresponding synthetic peptide, but failed to recognize the polyepitopes in flow cytometry and in ELISA (data not shown). The sera obtained after immunization of the peptide with the Leu-Asp spacer did not react either with the polyepitope, the corresponding peptide in flow cytometry and ELISA and was negative on MV. None of the sera obtained reacted with MV, indicating that neither of the peptides elicited MV cross-reactive antibodies.

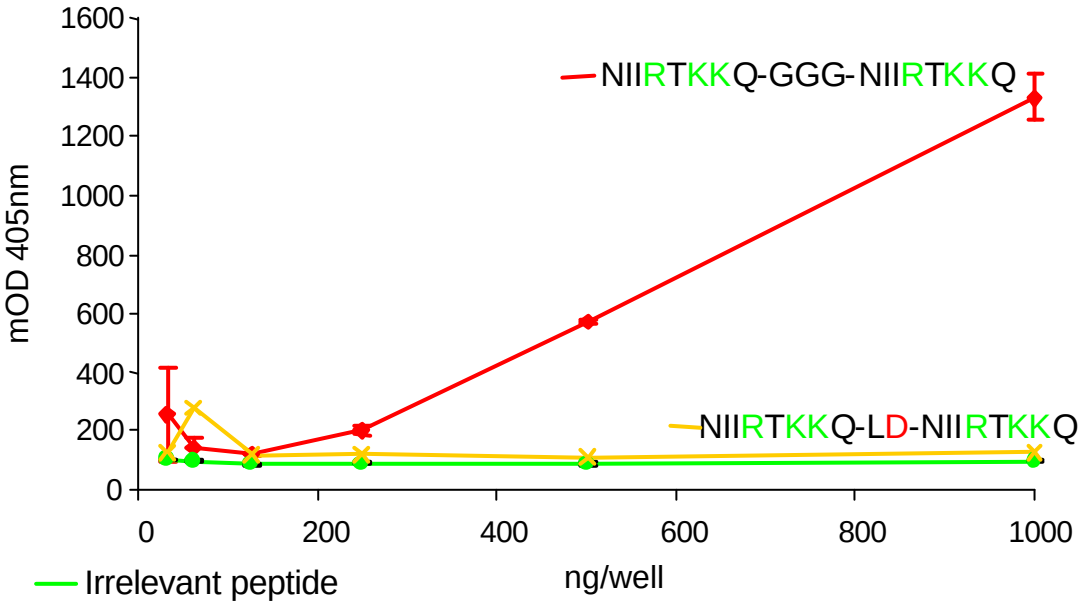

Fig 45: Reactivity of mAb F7-21 with mimotope peptide dimers

The ELISA test with the two synthetic peptides as antigens and the monoclonal antibody F7-21 (Fig 45) showed that this epitope-specific antibody is able to recognize the Mimotope-Gly-Gly-Gly-Mimotope whereas it did not detect the Mimotope-Leu-Asp-Mimotope peptide. This surprising result obtained with the monoclonal antibody and the polyclonal serum suggested that the LD spacer obliterated the antibody binding. The induction of a change in the peptide conformation and charge distribution, is likely to explain this effect.

III.2.2 Design and development of the antigen: Polyepitopes derived from antigenic regions

In this strategy, we used longer stretches of the 3 most immunogenic proteins of the measles virus, the hemagglutinin protein (MVH), the fusion protein (MVF) and the nucleoprotein (MVN). Different regions of the three most immunogenic measles virus proteins of the Edmonston strain (EMBL id: MV03669) have been selected for their content in B and T cell epitopes previously described in the literature (MVH, Table 8; MVF, Table 9 and MVN, Table 10).

On one hand, longer fragments of MVH proteins may improve the three-dimensional structure of the B cell epitopes which would have a greater chance to be conserved and stabilized by its natural flanking sequences. On the other hand, the conservation of the flanking regions of the T cell epitopes would potentially improve the generation of natural T cell epitopes during antigen processing. These long stretches of proteins may of course also contain important epitopes that have not been described.

The two surface proteins, but mainly the H protein, are the main targets of neutralizing and protective antibodies and provide the B cell epitopes for the constructs. The N and F fragments contain clusters of known T cell epitopes that are restricted by human and mouse MHC haplotypes. The inclusion in the chimeric constructs of TCEs which are potentially processed and presented by diverse human and murine MHC of class I and II should allow the testing of these constructs in vivo and in vitro in a mouse model and in vitro on human T cell lines. The CD8+ TCE on different N fragments should support the induction of a cytotoxic response in addition to the CD4+ restricted response. In fact, some studies (An and Whitton, 1997) have shown that CTL responses may, by themselves, give full protection.

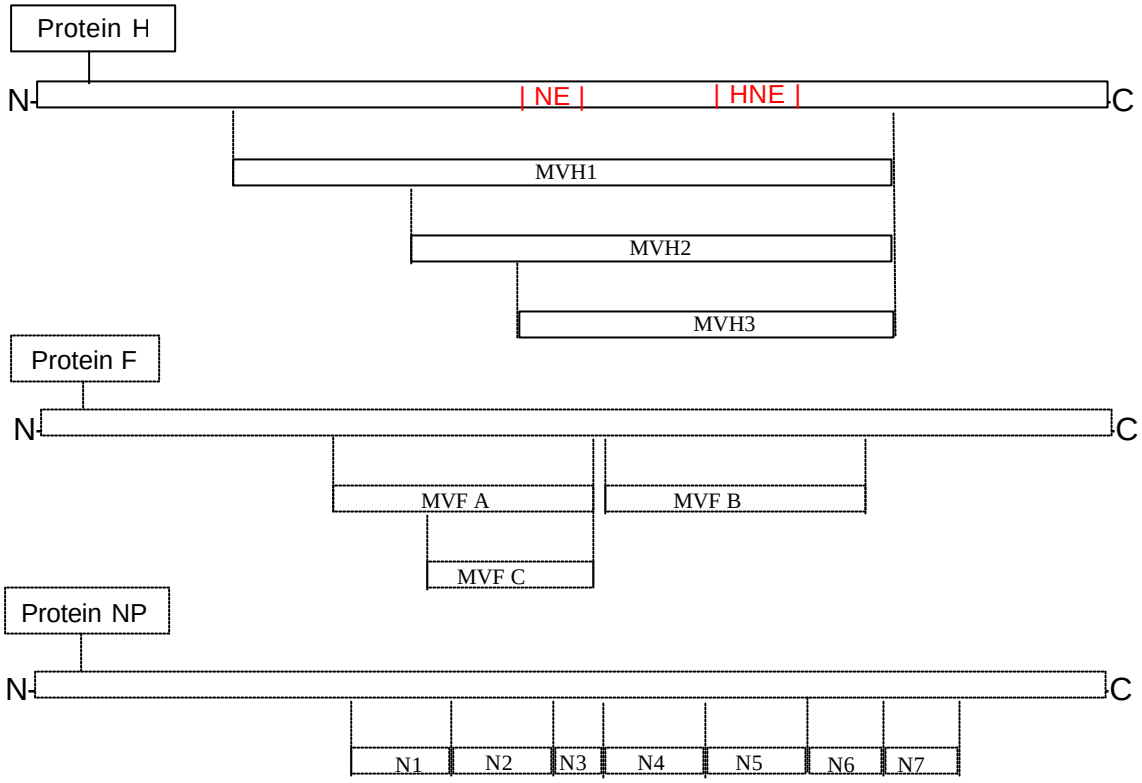

Fig 46: Position of the selected epitopes on the three MV proteins.

III.2.2.1 Description of the epitopes included in our strategy

III.2.2.1.1 H protein antigenic regions

Most of the protective activity of anti-MV antibodies is directed against the haemagglutinin protein (Giraudon and Wild, 1985; Sato *et al*, 1985), therefore it is not surprising that most of the neutralizing B cell epitopes have been described on this protein.

The MVH fragments MVH-1, MVH-2 and MVH-3 (Table 8) have been designed in order to contain several neutralizing BCE, which have been described in our laboratory using synthetic peptides, the NE (neutralizing epitope) and HNE (hemagglutinin noose epitope) (Fournier *et al*, 1997; El Kasmi *et al*., 1998), and as well as by other authors (Makela *et al*, 1989). The HNE and NE epitopes have been selected since they have been shown to induce protective antibodies in the presence of virus-neutralizing maternal antibodies (El Kasmi *et al*, 2001).

The selection of these antigenic regions of the H protein should allow the induction of virus crossreactive antibodies. In addition, the choice of sequences of different length (see Table 8) may also provide information on the sequence environment required for the display of these BCE in their natural conformation. In fact for some applications of this antigen, it may be interesting to include long sequences containing many T cell epitopes, but where the conformation of such epitopes would have little resemblance with natural protein and therefore resist neutralization by residual pre-existing antibodies. The DNA fragments encoding these protein regions were generated by PCR from the cloned cDNA or they were available as synthetic oligonucleotides.

Name	Sequence	Composition	Reference
MVH-1	125-410aa	125-135 : Neutralizing BCE	Makela *et al*, 1989
		185-195 : Neutralizing BCE	Makela *et al*, 1989
		236-255 : BCE (NE)	El Kasmi *et al*, 1998
		343-351 : Epitope CD8+, Ld	Beauverger *et al*, 1994
		379-410 : BCE (HNE)	El Kasmi *et al*, 1998
MVH-2	184-410aa	185-195 : B cell epitope, neutralizing	Makela *et al*, 1989
		236-255 : BCE (NE)	El Kasmi *et al*, 1998
		343-351 : Epitope CD8+, Ld	Beauverger *et al*, 1994
		379-410 : BCE (HNE)	El Kasmi *et al*, 1998
MVH-3	236-410aa	236-255 : BCE (NE)	El Kasmi *et al*, 1998
		343-351 : Epitope CD8+, Ld	Beauverger *et al*, 1994
		379-410 : BCE (HNE)	El Kasmi *et al*, 1998

Table 8: Known epitopes in the MVH protein fragments

III.2.2.1.2 F protein antigenic regions

Three antigenic regions of the F protein have been selected to be included in chimeric proteins. The choice of these subunits is based on their abundance in TCEs. The subunits have been obtained using PCR on a Bluescript vector containing the cDNA of the F protein.

Name	Sequence	Composition	Reference
MVF-A	197-341aa	199-228 : TCE, human promiscous	Muller *et al*, 1996b
		214-228 : TCE, DR1 restricted	Muller *et al*, 1995
		240-252 : BCE and TCE $H2^{s,k,a}$ restricted	Partidos and Steward, 1992
		256-270 : TCE $H2^{d}$ restricted (CD4+)	Muller *et al*, 1995
		258-277 : TCE $H2^{d}$ restricted (CD4+)	Stewart *et al*, 1995
		259-305 : TCE $H2^{d}$ restricted (CD4+)	Muller *et al*, 1995
		259-273 : TCE $H2^{d}$ restricted (CD4+)	Muller *et al*, 1995
		288-302 : TCE ($H2^{s,a,d,k,q}$ restricted)	Obeid *et al*, 1995
		291-306 : TCE mouse and human	Partidos *et al*, 1990
		317-329 : TCE, DR1 restricted	Partidos *et al*, 1991
MVF-B	381-451aa	391-405 : Neutralizing BCE.	Atabani *et al*, 1997
		399-423 : TCE DR2,DR7,DR3 restricted	Muller *et al*, 1996
		404-414 : Neutralizing BCE	Partidos *et al*, 1991
		408-418 : Non neutralizing BCE	Obeid and Steward, 1994
		424-438 : TCE, $H2^{d}$ restricted (CD4+)	Muller *et al*, 1995
MVF-C	251-341aa	256-270 : TCE $H2^{d}$ restricted (CD4+)	Muller *et al*, 1995
		258-277 : TCE, $H2^{d}$ restricted	Stewart *et al*, 1995
		259-305 : TCE, $H2^{d}$ restricted	Muller *et al*, 1995
		259-273 : TCE, $H2^{d}$ restricted	Muller *et al*, 1995
		288-302 : TCE ($H2^{s,a,d,k,q}$)	Obeid *et al*, 1995
		291-306 : TCE mouse and human	Partidos *et al*, 1990
		317-329 : TCE, DR1 restricted	Partidos *et al*, 1991

Table 9: Epitopes present of the MVF protein-derived fragments

III.2.2.1.3 N protein antigenic regions

After MV infection, the earliest and most abundant antibodies are directed against the nucleoprotein (Graves *et al*, 1984; Norrby *et al*, 1981), but they do not play a direct role in virus neutralization (Liebert and Finke, 1995), however they may enhance the processing of the N protein or even virus particles by faciliting antibody-receptor mediated uptake (Fournier *et al*, 1996). The N protein plays also an important role in the cell-mediated immune response (Bankamp *et al*, 1991; Rose *et al*, 1984).

The DNA fragments encoding seven antigenic regions, NP1 to NP7, were prepared using synthetic oligonucleotides. Given the length of each NP region, we decided to split the sequence into two

subfragments which were more easily synthetized. The fragment NP1 was split in NP1a and NP1b, for example. The oligonucleotides were designed in such a way that complementary single-stranded regions at the 3' end of the *a* fragments and at the 5'end of the *b* fragment allowed for the assembly of the double stranded *a* and *b* DNA without modification of the amino-acid sequence. The latter would have been the case if restriction sites were used for the assembly of *a* and *b*.

Name	Sequence	Composition	Reference
MVN-1	185-220aa	185-199 : TCE, DR5 restricted. 210-218 : CTL, HLA-A2 restricted.	Nanan *et al*, 1995
MVN-2	221-256aa	221-240 : TCE, DR restricted. 226-234 : CTL, HLA-A2 restricted. 237-256 : CTL, DR restricted.	Hickman *et al*, 1997 Nanan *et al*, 1995 Hickman *et al*, 1997
MVN-3	281-290aa	281-290 : CTL, Ld, CDV cross-reactive.	Beauverger *et al*, 1993
MVN-4	329-366aa	329-345 : TCE, $H2^d$ and Iad restricted 340-348 : CTL, HLA-A2 restricted	Fournier *et al*, 1996 Nanan *et al*, 1995
MVN-5	367-420aa	367-386 : TCE, DR restricted 377-399 : TCE, $H2^d$ 400-420 : TCE, DR restricted	Hickman *et al*, 1997 Fournier *et al*, 1997 Hickman *et al*, 1997
MVN-6	445-475aa	445-465 : TCE, $H2^b$ 457-476 : BCE	Giraudon *et al*, 1988
MVN-7	476-507aa	483-502 : TCE, DR restricted	Hickman *et al*, 1997

Table 10: Epitopes present of the MVN protein-derived fragments.

III.2.2.2 Design and assembly of the constructs

Two different sets of constructs (Table 11) were assembled, which contained either A+B or the C+B segment of the MV-F protein. These constructs would allow to study the immune response to H protein antigenic fragments in a recombinant construct containing the same T cell determinants. The N protein is a source of MHC-I epitopes, providing an activation source for CD8+ lymphocytes.

Fusion protein (T cell epitopes)	Nucleoprotein (T cell epitopes)	Hemagglutinin protein (B cell epitopes)	Used name
MVF (A+B)	MVN (1+2+3+4+5+6+7)	MVH-1	AB MVH1
		MVH-2	AB MVH2
		MVH-3	AB MVH3
		No MVH fragment	AB NP1-7
MVF (C+B)	MVN (1+2+3+4+5+6+7)	MVH-1	CB MVH1
		MVH-2	CB MVH2
		MVH-3	CB MVH3

Table 11: Different types of constructs designed

This new set of constructs was built into a pUC18 vector using a modified multiple cloning cassette (START Cassette) containing the restriction sites for transfer into a bacterial expression vector.

The oligonucleotides were first annealed and each subunit was cloned into a separate pUC start vector, generating the subunits vector pUC MVH1, pUC MVH2, pUC MVH3 for the subunits derived from the H protein, pUC MVF-A, pUC MVF-B, pUC MVF-C for the F protein and pUC MVN-1, pUC MVN-2, etc, for the subunits of the N protein. These vectors were used to transform DH5α bacteria. The transformed bacteria were plated on agar plates containing ampicillin. DNA was extracted from the resulting colonies and digested using restriction enzymes to obtain size-discriminating DNA fragments. The length of the digested fragments obtained after digestion was checked on an acrylamide gel. The best clones of every subunit were selected and further tested by sequencing. We proceeded to the next step only after ensuring the perfect conservation of the cloned sequences and the multiple cloning sites. The subunits of each protein were then cloned into the above pUC START vector. The result of each successive cloning were confirmed by enzymatic digestion and sequencing. The sequences of the original measles genes were finally assembled into the 6 structures described in the Table 11. The final constructs were confirmed again by sequencing.

During the above series of repeatitive cloning steps (which were the longest and most critical series of experiments in the present thesis), intermediate steps were kept as references and could be used as controls during later experiments or in future projects because of their compatibility with other cloning strategies.

The set of constructs were then transferred to the modified pET vector used for expression. A final sequencing ensured that the transfer phase was done without altering the sequence. The colonies carrying the constructs were then amplified and stored as glycerol stocks.

III.2.2.3 Optimization of the induction of MVH1 constructs

The T7 system was used to express the constructs described above. In this experiment, we have tested induced cultures of MVH1 subunit, as well as AB NP1-7 and AB MVH1 constructs. Non-induced AB NP1-7 cultures and induced empty vector (pET16pelB) were included as negative controls. After induction, the bacterial pellet has been lysed using the osmotic shock protocol described in the pET manual (pET system manual, 7th edition, Novagen, USA). The proteins contained in the periplasmic fraction have been coated in a 96-well plate and tested in an ELISA test using anti-H Mab BH195 (dilution 1:1000) which would be expected to react with the epitope contained in this fragment. The Fig 47 shows low but significant signals (<0.2 OD) for MVH1 and AB MVH1 constructs. This indicated a weak expression of the relatively short MVH1 construct and a very weak or negative expression of the other constructs. The detection level of of AB NP1-7, containing no MVH fragments, was equal to the non-induced cells with pET AB NP1-7 vector or cells with an empty pET vector, indicating low but specific detection of the Mab.

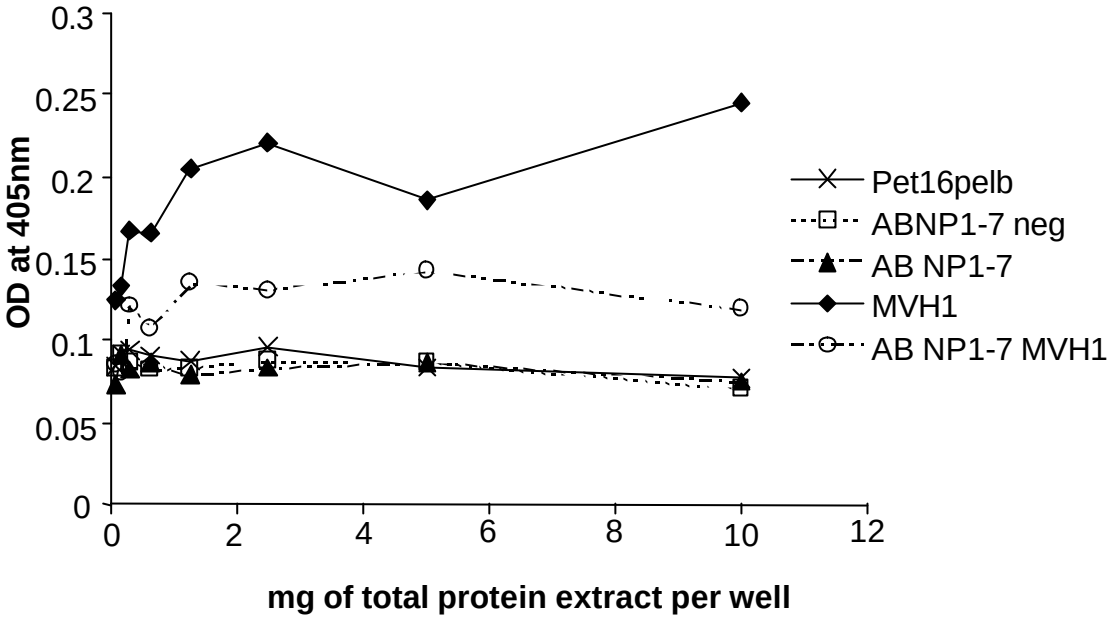

Fig 47: Detection of constructs using anti-MVH Mab BH195

Such a low expression can be explained by a low efficiency of induction. In the induction protocol described in the Material and Methods, several factors could potentially be improved, including:

- Duration of induction: A longer duration would allow more time for the BL21DE3 cells to produce the heterologous proteins. The time indicated in the protocol may be suboptimal for the size of the proteins investigated here.

- Concentration of IPTG (isopropyl-D-thiogalactopyranoside): Higher concentrations of IPTG could possibly recruit more polymerase. On the other hand, larger constructs may also require more time for translation and higher concentrations of polymerase may have no effect or even a negative effect. Different concentration of IPTG during induction will be tested.

- Repetitive pulses: Data about the rate of metabolization of the IPTG are difficult to obtain and there is no information on whether it may be more efficient to deliver a higher dose of IPTG to start the induction or to provide lower but repetitive doses of IPTG during the whole induction process. Different patterns of IPTG delivery will be investigated.

- Reassessment of purification methods: the heterologous proteins might have been produced in reasonable amounts but the purification efficiency may have been low, so different means of purification procedures including size exclusion (Amicon 10kd filters) and affinity purification using monoclonal antibodies and/or his-tag purification will be explored. A poly-histidine coding sequence was cloned at the C-terminal end of every

construct to avoid the co-purification of incompletely synthesized or degraded proteins. Since the poly-histidine tail is produced last, incomplete proteins would not bind to the nickel-loaded resin during purification.

III.2.2.3.1 Duration of induction

To optimize the effect of the duration of induction, a bacterial culture of BL21DE3 cells transformed with a pET vector containing the AB NP1-7 MVH1 gene was initiated. When the culture reached 0.4 OD at 600nm, it was splited into 4 sub-cultures of equal volumes. To each sub-culture 1mM final concentration of freshly prepared IPTG was added and returned to 37°C under agitation. One culture was removed after 2 hours of induction, a second bottle after 8 hours, a third after 12 hours and the last one after 20 hours of induction. The cultures were centrifugated and lysed following the standard protocol.

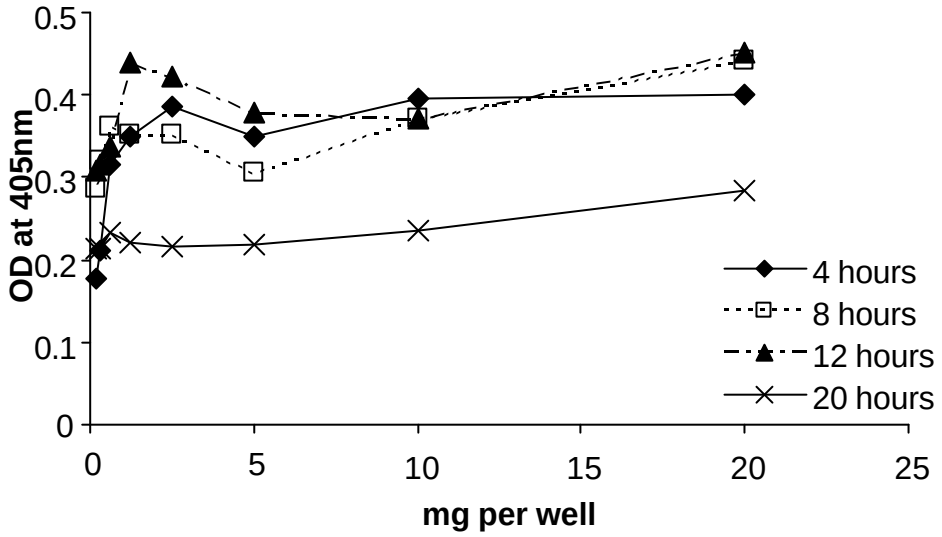

Fig 48: Detection of ABNP1-7 MVH1 using anti-his Mab following different times of induction

Fig 48 shows that maximal levels of proteins were already obtained within 4 hours of induction. After 20 hours and more, the efficiency seemed to decrease. This may be due to protein degradation by bacteria that suffered from increasing toxicity of the accumulating protein and/or exhaustion of the culture medium. The optimal time of induction seems to be in the 4-12 hours bracket.

III.2.2.3.2 Concentration of IPTG

When the cells were incubated with increasing concentrations of IPTG, it became apparent that IPTG had an effect on cell growth, measured by optical density at 600nm. The recruitment of cell ressources towards protein production at the expense of cell growth and division could be an explanation for this observation. While this could be beneficial, it may also affect the overall efficiency of protein production negatively as a result of lower cell numbers. A compromise between single cell efficiency and overall efficacy of all cells is required.

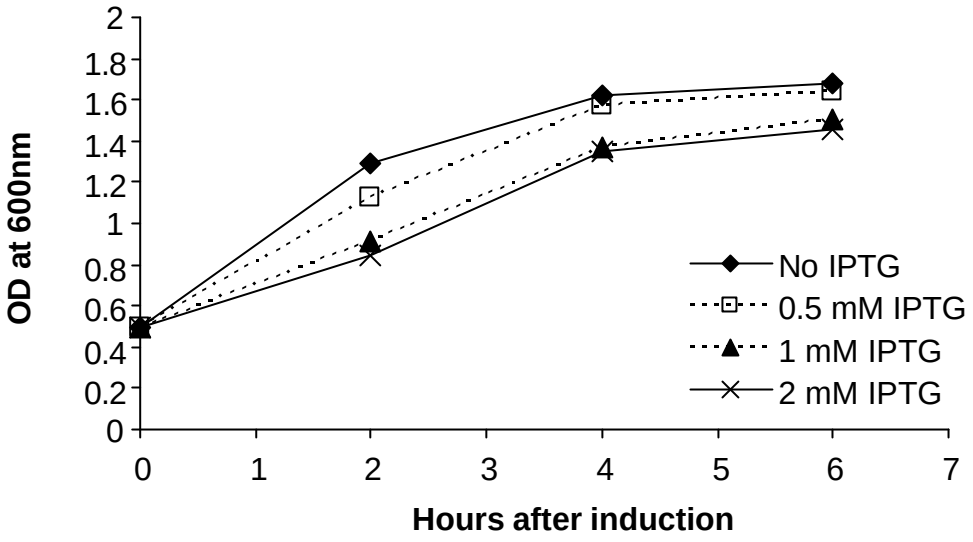

Fig 49: Effect of IPTG concentration on cell growth

III.2.2.3.3 Effect of repetitive pulses of IPTG

In this experiment, IPTG was provided to two bacterial cultures derived from a single overnight culture. The first culture was supplemented with 2mM of IPTG at time 0 and was harvested 4 hours later. The second culture was treated with the same total amount of IPTG but given in two separated doses at 0 and 2 hours.

Fig 50 shows that under the two-dose schedule, the expression of the culture induced with two pulses of IPTG was improved by about 20%. Based on these results, 1mM IPTG was added every 2 hours to the culture of the following experiments and 4 to 8 hours of induction was considered optimal.

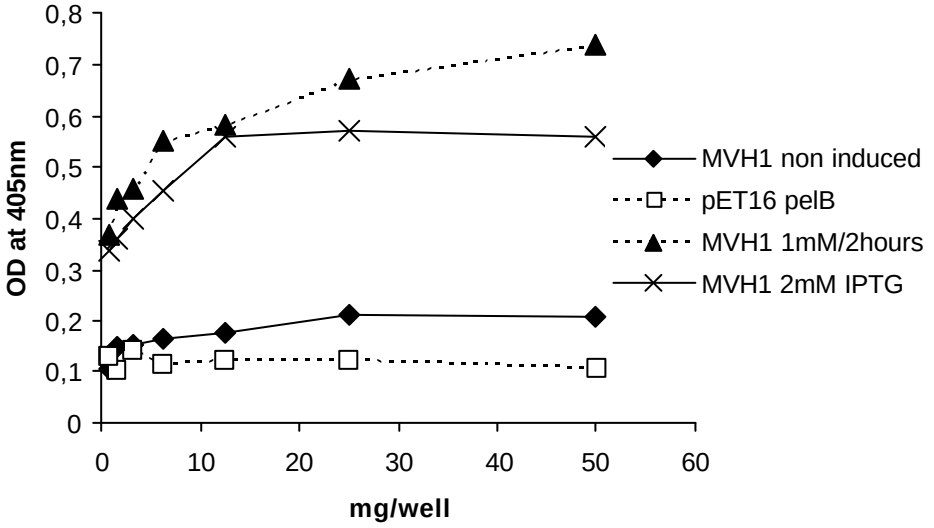

Fig 50: Effect of repetitive pulses of IPTG detected using anti-MVH antibody BH195

III.2.2.4 T cell stimulation assay

III.2.2.4.1 T cell stimulation assay using T cell hybridomas

The T cell assay was used both to detect the chimeric protein and to show that the protein can stimulate antigen-specific T cells. Furthermore antibodies specific for B cell epitopes of the constructs were used to test whether they would enhance antigen uptake. This T cell proliferation assay uses mouse T cell hybridomas which produce IL2 upon stimulation. After incubation of antigen and APCs with the T cell hybridomas, the supernatant was transferred to plates containing IL2-dependent T cells (CTLL). The IL2 that was produced during priming of the T cell line induces the proliferation of the CTLL cells which is measured by WST1 absorbance. WST1 is cleaved by mitochondrial dehydrogenase in viable cells and produces a red color that can be quantified by reading 96-well plates at 450nm.

The T cell assay was performed using cell line M12.4.1 as antigen presenting cell [B cell lymphoblastoma in (Kim *et al*, 1979)]. Two mouse T cell hybridoma lines, TNP79 (stimulated by epitope NP330-345) and TNP408 (stimulated by the epitope NP380-395) were used since these two epitopes are present in the constructs. Due to the relatively low sensitivity of T cell hybridomas in comparison with human T cell lines, antigen uptake was enhanced by incubating the constructs with a set of antibodies at a final concentration of 100ng per ml and per antibody.

The antibodies used were:

- BH47 (specific of the NE region of the H protein: aa236-255)
- BH195 (specific of the HNE region of the H protein: aa 379-410)
- BNP146 (directed to the 376-395 region of the nucleoprotein)
- BNP176 (directed to the 136-144 region of the nucleoprotein)

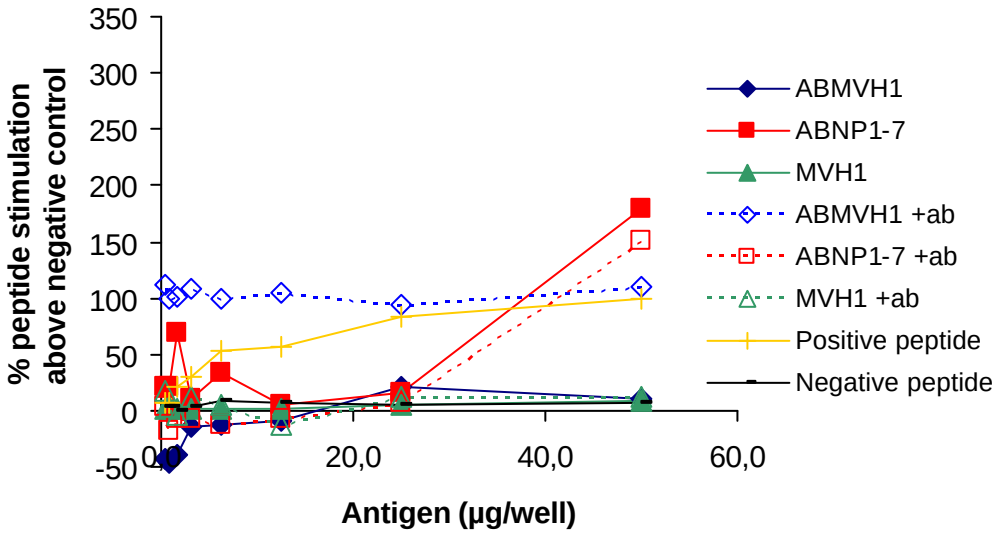

Fig 51: Stimulation of TNP79 hybridomas induced by our constructs with and without incubation with antibodies

Fig 51 shows that the constructs ABNP1-7 induced a significant IL2 production at the highest concentration (50µg of total protein). ABNP1-7 stimulated the T cells in a dose-dependent manner and the addition of antibodies did not enhance antigen presentation. As expected, MVH1 constructs did not stimulate the T cells, with and without antibodies, as no stimulating epitopes are present in this construct. Conversely, ABMVH1 (containing the same NP fragments than ABNP1-7) did not stimulate T cells and the addition of antibodies only produced non-specific dose-independent stimulation. CBMVH3 could not be tested in this assay because it induced cell death as observed under the microscope.

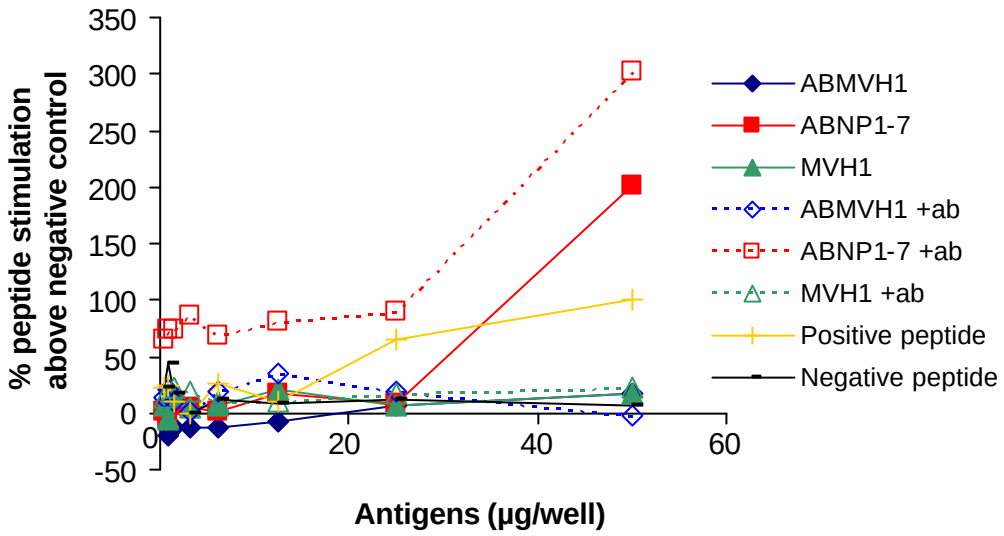

Fig 52: Stimulation of TNP408 hybridomas induced by our constructs with and without incubation with antibodies

The constructs also contain the epitope N380-395 of the T cell hybridoma TNP408. Fig 52 shows that again ABNP1-7 stimulates at the highest concentration and that the addition of monoclonal antibodies enhanced antigen presentation, but the antibody also increased unspecific stimulation. The response to MVH1 and ABMVH1 was negative irrespectively of the presence or absence of monoclonal antibodies. As with the TNP79, we observed cell death induced by CBMVH3.

III.2.2.4.2 T cell proliferation assay using human T cell line

In this experiment, specific human T cell lines were used to test the ability of the chimeric constructs to induce T cell proliferation. In general, human T cells tend to be more sensitive than murine T cell hybridomas.

Four different T cell lines were tested: two cell lines were specific for the epitopes 254-268 of the F protein (cell line FR6.6 and 254T2) but these two F-specific cell lines were not stimulated by either the constructs containing the epitope or the positive control (data not shown). They were therefore excluded from the study. Cell line NP1.5 is specific of epitope 185-199 of the nucleoprotein. In addition, TT830 T cell line, specific of the tetanus toxoid, was used as a negative control.

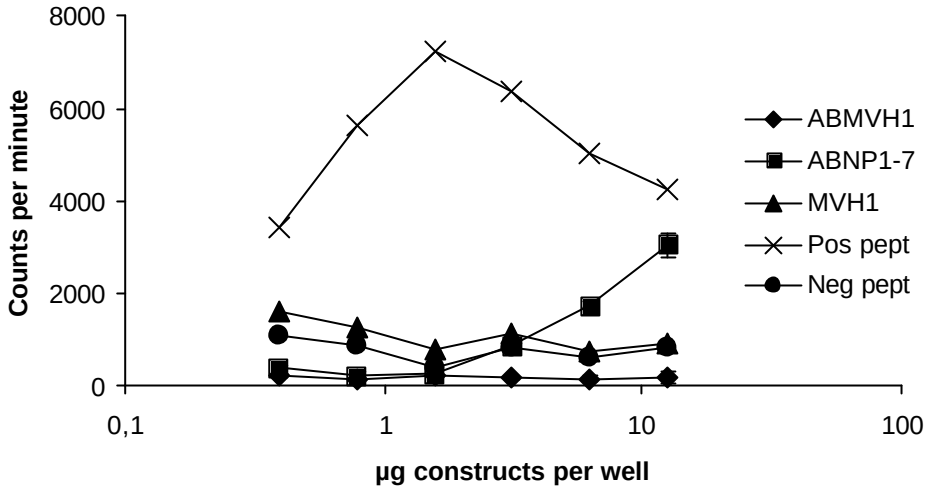

Fig 53: Stimulation of T cell line NP1.5 induced by our constructs

Fig 53 shows that ABNP1-7 induced a proliferation of the NP1.5 cell line, with a maximum stimulation at 12 µg per well. Higher concentration of the constructs seemed to be toxic to the cells, as observed in previous experiments for the CBMVH3 (data not shown). MVH1 (that does not contain the specific T cell epitope) has a pattern similar to the negative peptide and is thus considered negative. The ABMVH1 constructs is completely negative over the whole range of concentration, perhaps because of its low concentration in the antigen mix.

The TT830 T cell line was used to monitor non-specific proliferation. As expected, this cell line reacted with its specific peptide but was negative for all the investigated constructs (Fig 54), none of which contained the tetanus toxoid T cell epitope.

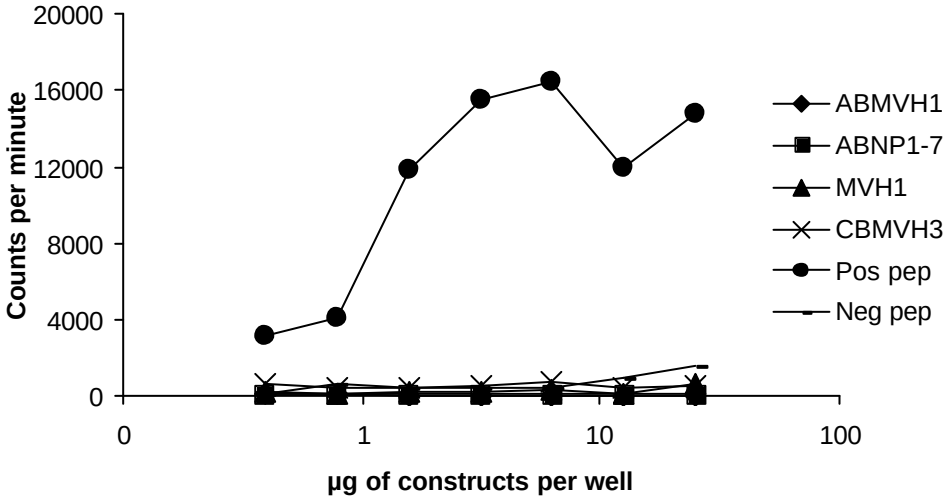

Fig 54: Stimulation of T cell line TT830 induced by our constructs

III.2.2.4.3 T cell proliferation assay using human T cell lines and antibody priming

In the previous experiments, mytomycin C was used to prevent the proliferation of the APC during the experiment. The previous experiments raised the question whether mytomycin C could interfere with the processing of the longer constructs, by inhibiting the synthesis of proteosomes or MCH molecules (Tomasz *et al*, 1987; Rahmsdorf *et al*, 1986). Therefore, the experiment of Fig 53 was repeated with NP1.5 human T cell line, adding mytomycin C only 5 hours after antigen pulsing.

Fig 55 shows that this experiment essentially confirms the results of the previous ones.

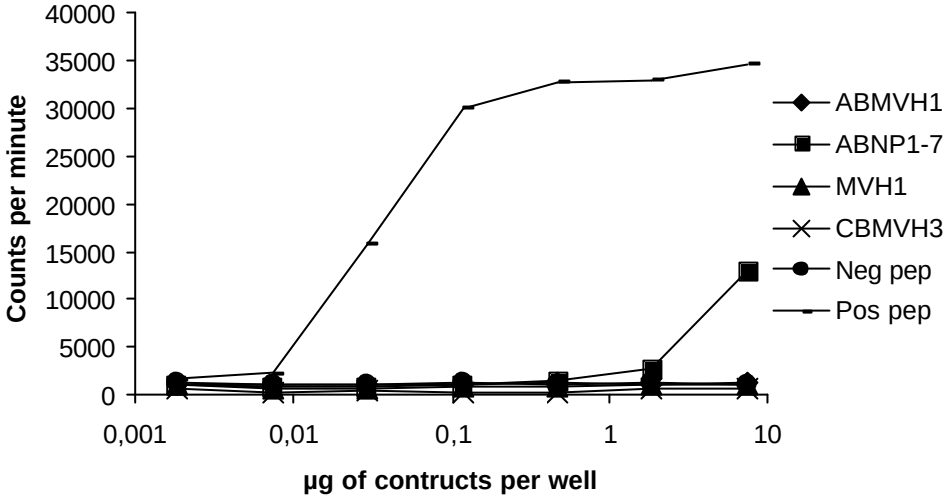

Fig 55: Stimulation of T cell line NP1.5 induced by our constructs, using primed APC

III.2.2.5 Immunogenics studies

To investigate if mouse serum obtained after immunization with different constructs contained cross-reactive antibodies, sera were tested by flow-cytometry against MV-infected WMPT cells. In this assay, anti-MV antibodies would bind to the MV-glycoproteins budding from the cell surface.

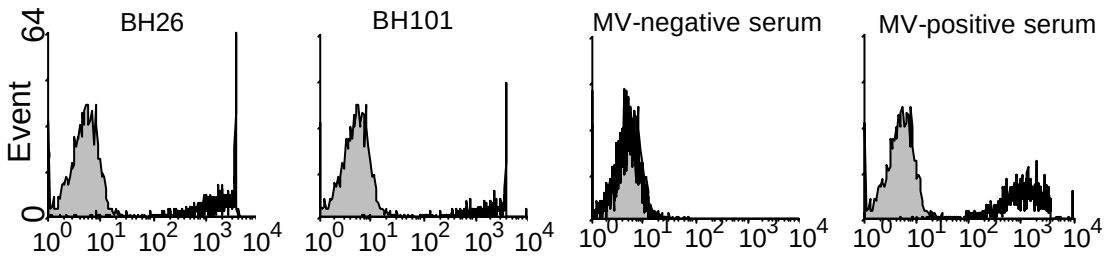

Fig 56: Controls of the WMPT experiments.

In Fig 56, the MV-specific mabs BH26 and BH101 show a very strong expression of hemagglutinin protein associated with budding MV (in fact, the signals are virtually off-scale). In contrast, uninfected cells are negative with the same mab. A serum from an MV-positive individual binds to the MV surface protein and the serum of a naïve individual did not generate a positive signal.

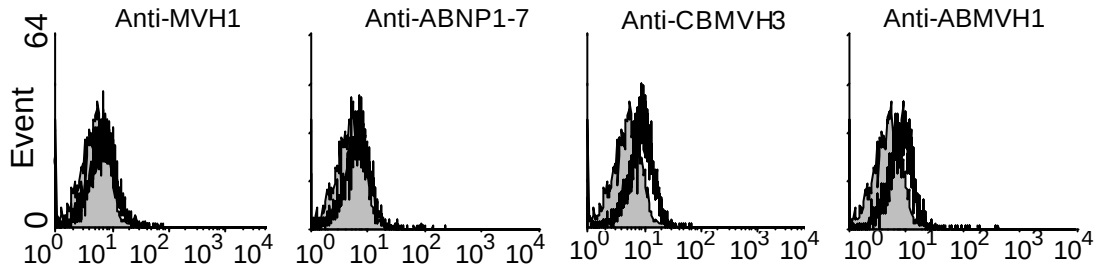

Fig 57: Histograms of the WMPT cells reacted with sera from mice immunized with the MVH1, ABNP1-7, CBMVH3 and ABMVH1 proteins. The gray histogram shows the un-infected WMPT cells and the black bold line shows the results obtained with the MV-infected WMPT

Fig 57 shows only very weak or negative reactivity. This would suggest that none of our constructs elicited antibodies cross-reactive with the two surface proteins MV-F or MV-H.

The sera were also tested by ELISA to confirm that the constructs induced antibodies.

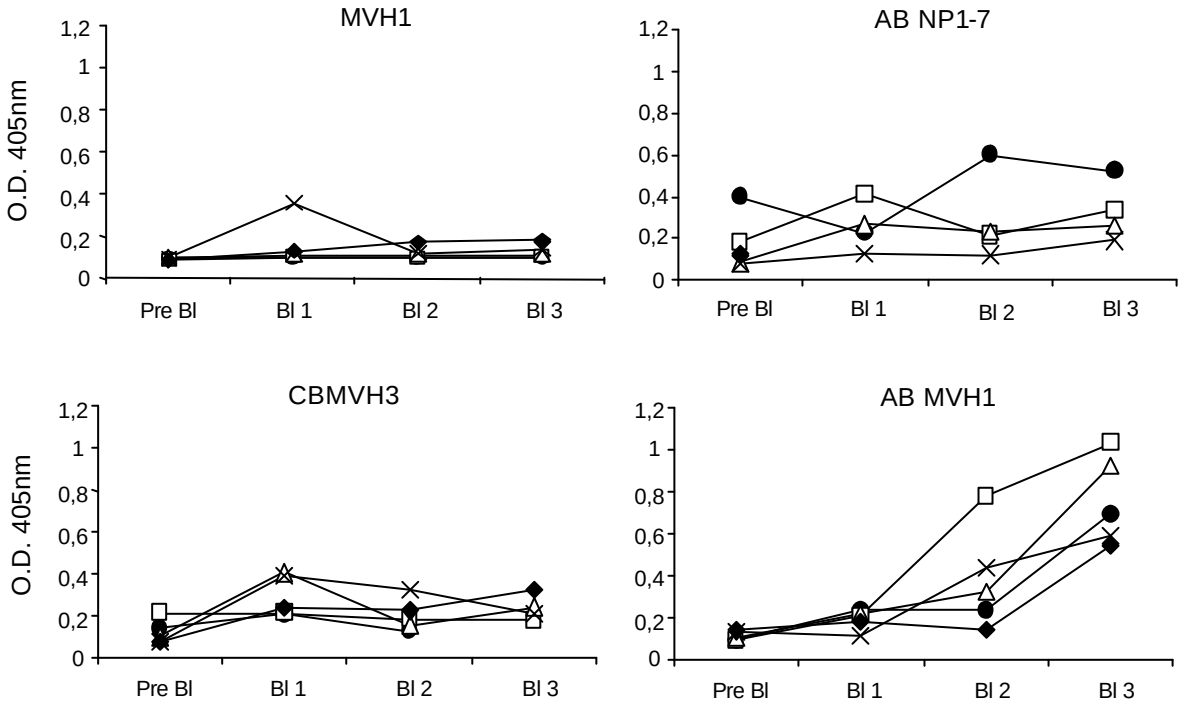

Fig 58: Response of the mice sera to the Measles virus.
The graphs show the OD values at 405nm of the 5 individual mice of each group compared with the different blood samples. Pre-bl, bl1, bl2 and bl3 stand respectively for pre-bleeding, bleeding after boost 1, bleeding after boost 2 and bleeding after boost3

Fig 58 shows that the sera obtained after immunization with ABMVH1 react strongly with coated measles virus. Sera obtained with ABNP1-7 reacted weakly after the third boost. This suggests that either the FACS experiment was not very sensitive or that the ELISA detected antibodies directed

against the nucleoprotein. The construct ABNP1-7, that stimulated T cell proliferation, also induced detectable amounts of anti-MV antibodies. Although construct ABMVH1 gave only very weak reactions in the T cell assay, the mouse sera reacted with the antigen lysate in ELISA.

The above results showed that although overall expression of the constructs in bacteria was probably low, some chimeric proteins induced antibodies that cross-reacted with coated measles virus in ELISA. However, when the same sera were tested against measles virus budding from the surface, they were negative. These two results can be reconciled if one considers that a high proportion of the coated MV in ELISA is denatured (Worldwide vaccines, 2002) and it is probably this fraction that is recognized.

Part IV

Discussion

IV. DISCUSSION

IV.1 Secondary immune response in adults with low levels of antibodies

There are different manifestations after contact with wild-type measles depending on the pre-existing immune status (Aaby *et al*, 1986; Pedersen *et al*, 1989). Seronegative people generally develop clinical measles. While seropositivity normally protects against disease, this study demonstrates that a SIR occurs in individuals protected by wild-type virus induced immunity. Among 45 fully protected, measles late convalescent parents reexposed to measles, 4 developed an asymptomatic SIR with a significant increase in MV-specific IgG. Mainly the IgG1 subtype was boosted, which is also the predominant subclass during primary infection (Mathiesen *et al*. 1990). SIR included antibodies against MV-H and against MV-NP, which are also the main antibody targets during primary response (Norrby *et al*, 1981). SIR was sometimes associated with a weak IgM response as reported before (Murray and Lynch, 1988; Sekla *et al*, 1988). Higher and more transient increases of IgM may have been missed in sera which were drawn later after exposure.

SIR was only found in individuals with a pre-exposure IgG level below a well defined threshold which was determined in this study to be IgG<780-850 mO.D., NT<1:$2^{5.5}$-$2^{6.5}$, HI<1:$2^{6.5}$-2^{7}. Above this threshold none of the parents developed a reaction to MV after exposure. SIR seems to be an "all or none response" where the magnitude of increase in specific IgG, NT and HI is independent of pre-exposure antibody levels as long as these are below a certain threshold (Muller *et al*. 1996). However, a single serological parameter may not be sufficient for the assessment of SIR susceptibility. The four parents with SIR were the only individuals which were below the threshold for the three parameters, suggesting that only low specific IgG combined with low NT and HI titers predispose for SIR.

Another observation was that antibody levels tended to decrease again after exposure. Antibodies persisted, however, long enough that an IgG increase would not have been missed in the other sera taken 4.6-6 weeks after exposure. This observation suggests that the low antibody levels found in these individuals are not caused by an insufficient MV contact, but rather to a low-responder status, which may be genetically defined as has been suggested (Poland *et al*, 1995; Hayney *et al*, 1997). Consenting individuals with short-lived SIR could potentially be useful for the surveillance of circulating virus. A similar short-lived antibody increase was also observed in vaccinees after booster immunisations with live measles vaccine (Markowitz *et al*, 1992).

Several observations indicate that measles virus can circulate among seropositive persons (Pedersen *et al*, 1989; Gustafson *et al*, 1987). In a secluded population of seropositive vaccinees, most experienced an increase in measles titers several years after vaccination. In the absence of clinically overt measles, this suggested that the virus circulated also in healthy vaccinated individuals which could potentially transmit disease to seronegative people (Pedersen *et al,* 1989).

It is likely that this requires a transient viraemia. People undergoing a clinically inapparent SIR are the most likely candidates to support such a transmission of virus. In this context, the transient nature of the SIR seems to indicate that such individuals can be efficiently protected from disease but not necessarily from infection. The frequency of SIR during measles outbreak was about 5% (Ozanne *et al*, 1992; Edmonson *et al*, 1990), however, the number of susceptibles to SIR may be considerably higher. Our data can be used to estimate the frequency of susceptibles on the basis of their pre-existing IgG, NT and HI levels. Such estimates may become important, since after vaccination antibody titers are lower and more likely to wane than after wild-type infection (Weibel *et al*, 1980; Krugman, 1983; Pedersen *et al*, 1986; Christenson and Böttiger, 1994). Therefore, with more and older vaccinated people the number of susceptibles to SIR is likely to increase in the future.

Our study suggests that a subset of individuals which may be transiently contagious, can be serologically identified on the basis of their pre-existing immunity and that the estimation of their frequency could be relevant for future MV epidemiology and vaccination strategies. In only one case, wild-type virus has been isolated from individuals with asymptomatic secondary immune response (Kreis and Schoub, 1998), possibly because they are poorly defined. But direct case confirmation of unapparent secondary immune response is also complicated by the need of documented measles virus contact as well as pre- and post-re-exposure serum samples. It is therefore difficult to obtain direct estimates of the frequency of persons susceptible to asymptomatic secondary immune response.

In this view, the study of other representative populations could shed light on the percentage of SIR-susceptibility in the population. We assessed here the fraction of individuals which would undergo SIR when in contact with a measles patient. This estimate is based on the pre-reexposure serology of parents from measles children undergoing an asymptomatic SIR with concomitant increase in specific IgG, NT and HI titers defined earlier.

In a population of 277 adults with natural immunity after measles infection, 3.2-3.9% exhibited the pre-reexposure serological characteristics of the SIR-competent parents. Among vaccinated children, up to one-third (22.2-33.2%) seemed susceptible to develop SIR upon contact with a measles patient, no matter if they were vaccinated once or twice. Christenson and Böttinger (1994) showed that specific titers can be boosted by revaccination, while boosting late convalescent individuals was not possible, demonstrating that vaccines are more likely to undergo vaccine-induced secondary immune response than late convalescents. In our study, SIR susceptibility was not reduced by a second vaccination, although revaccination reduces susceptibility to measles (Hutchins *et al*, 1990; Robertson *et al*, 1992; Tulchinsky *et al*, 1993).

SIR may present with unspecific symptoms, mild or even typical measles (symptomatic SIR) (Aaby *et al*, 1986; Edmonson *et al*, 1990) or it may remain clinically silent (Pedersen *et al*, 1989). It

appears that asymptomatic SIR may be a serological reaction to MV re-exposure detected in seropositive individuals protected against disease.

	SIR Susceptibility	
Cohorts	Minimum (%)	Maximum (%)
Early convalescents	0	0
Late Convalescents	3.2	3.9
Vaccinated once	22.2	33.2
Vaccinated twice	32.6	32.6

Table 12: Estimated frequency of SIR-susceptibles among early and late convalescent donors and vaccinated high school children.

The above estimates include seronegative individuals susceptible to disease and those prone to develop asymptomatic SIR.

In the first instance, among the vaccinated children 3-4% were seronegative by ELISA, but less than half of these were also negative for HI and NT. Such seronegative vaccinees correspond to primary vaccine failures and must not be counted as susceptible to SIR, since they would develop disease upon measles exposure. These values are in agreement with the primary vaccine failure rates of other studies (Miller *et al*, 1995). In the late convalescent cohort, <1% is seronegative, i.e susceptible to clinical measles.

Symptomatic SIR is mainly seen as secondary vaccine failure and is only rarely found in measles convalescents (Aaby *et al*, 1986; Edmonston *et al*, 1990; Miller *et al*, 1995). Most estimates of secondary vaccine failure range from 0% (Gustafson *et al*, 1987; Anders *et al*, 1996) to about 5% of either the measles patients or the vaccines (Edmonston *et al*, 1990; Ozanne and d'Halewin, 1992). Measurements of symptomatic SIR susceptibility in a population largely depend on MV exposure and on the sampling method.

Even when primary and secondary vaccine failures are taken into account, our data still suggest that a large proportion of vaccinees is susceptible to asymptomatic SIR. Susceptibility to unapparent SIR may be 5 to 8 times higher than to symptomatic SIR. In a fully vaccinated population asymptomatic SIR was found to be as high as 66% (Pedersen *et al*, 1989). That study describes a population with a high risk for SIR, in which viral replication could potentially be studied after measles exposure. Moreover, serological follow-up of voluntary contacts between SIR-susceptibles and accidentally measles exposed secondary immune response susceptibles could shed light on the potential infectivity of individuals undergoing secondary immune response.

As a result of sustained vaccination programs, measles immunity will wane in the general population and will eventually fall to the level of vaccine-induced immunity.

A simple estimation (Fig 59) shows the status of the population and the evolution of SIR susceptibility in a population. If we consider that in 1975, the whole population was protected by natural infection, the proportion of SIR susceptible would be equal to the late convalescent cohort (approx 3.2 – 3.9%). A population that is protected only by vaccine-induced immunity (100%) would have a proportion of SIR equal to the cohort of vaccinated children (22.2 – 33.2%). As the percentage of individuals protected by vaccination in the Luxemburgish population is about 32%, we could postulate, using a linear relation, that the current proportion of SIR susceptible is about 10%.

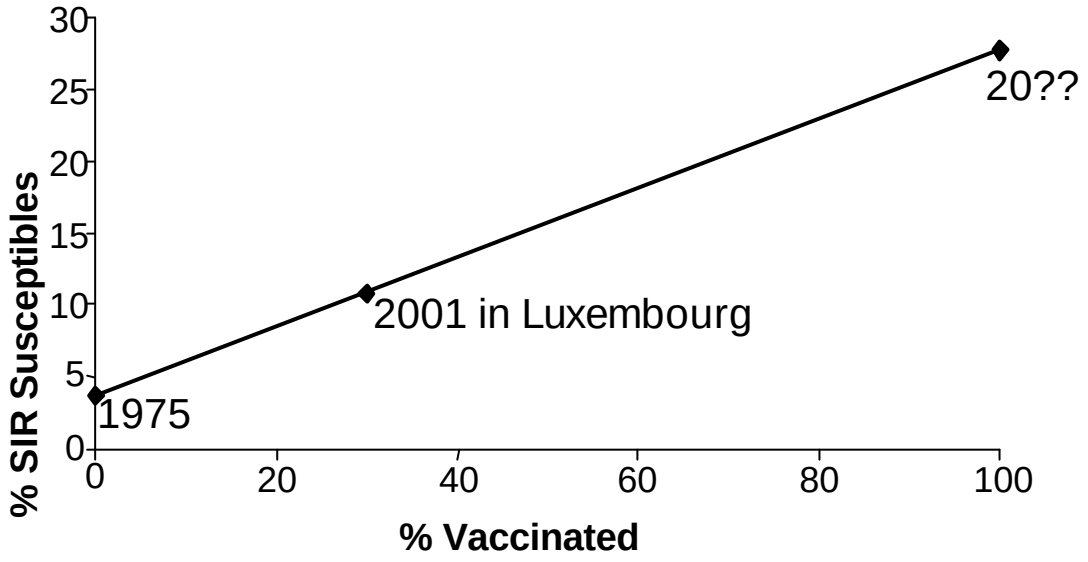

Fig 59: Estimation of the fraction of SIR susceptible in the population

Moreover, in the elderly, vaccine-induced immunity wanes faster than natural immunity (Markowitz *et al*, 1990; Christenson and Böttiger, 1994), meaning that the proportion of SIR susceptible in a population vaccinated to 100% could be potentially much higher that in our estimation. A study based on a similar data set (Mossong *et al*, 1999) has used a mathematical model to determine that after 25 years, 50% of seroconverted vaccinees would have an antibody titer below protective level. This means that 80% of all vaccinees will eventually loose protective levels of antibodies. In this case, elimination of measles virus cannot be achieved by a single-dose routine vaccination strategy if the basic reproduction number of vaccinated SIR-susceptibles exceeds 1.24 (Mossong *et al*, 1999).

If SIR susceptibility plays a role in the epidemiology of measles, this role is likely to grow in fully vaccinated populations where SIR could affect 30 % or more. Vaccination strategies as well as global eradication programs may have to consider the increasing number of SIR susceptibility in

highly vaccinated populations, and this in addition to enhanced susceptibility due to waning immunity.

The duration of the “booster effect”, the antibody increase detected in the SIR parents, can also be an issue in the development of further strategies. Indeed, 30±1 week after MV-reexposure, 10-25% of the increase in specific antibody was already lost, with similar decrease in HI and NT. This demonstrates that the booster effect was only transient. Although none of the parents have already recovered their pre-exposure immune status, the antibody titers are coming close to the threshold values (780-870mOD) and are still decreasing 4 years after exposure. It is possible that the SIR parents may become SIR-susceptible again and therefore could again be transiently infected by measles virus.

IV.2 The vaccine strategies

Our concept of a subunit vaccine consists in the design of a recombinant protein containing immunologically relevant peptide sequences derived from one or several pathogens.

Two complementary strategies, which differ mainly by the size of the pathogen-derived peptide sequences, have been used. One is based on the use of minimal epitopes allowing the incorporation of a maximal number of immunologically relevant peptides into a small protein, but using no human T-cell epitopes to prevent any risks of atypical disease. The other strategy uses larger pathogen protein fragments, containing BCE and TCE in their natural local sequence environment, with the objective to boost the immunity of vaccinated individuals.

In both cases, DNA fragments, encoding the BCE and TCE peptides, are assembled using molecular biology techniques to generate artificial genes encoding recombinant proteins differing by the order, orientation and nature of the subunits. These DNA constructs will subsequently be expressed in eukaryotic or prokaryotic cells. The recombinant proteins will be isolated and used for testing their antigenicity and immunogenicity. We designed our polyepitopic constructs by molecular biology techniques, because this technique allows to create longer constructs than with peptide synthesis. Moreover, as the constructs are made of linear epitopes or mimotopes, they may not require a defined tridimensional structure for recognition by immunoglobulins. Therefore, these artificial proteins may have the advantage of being more stable.

IV.2.1 Polyepitopes based on mimotopes

Routine vaccination against measles in young infants is performed at 15 months of age in the developed countries and at 9 months in the developing world. Earlier vaccination is usually associated with a low seroconversion rate due to persisting transplacentally transferred maternal antibodies (Albrecht *et al*, 1977). These antibodies neutralize the low dose of live viruses contained in the attenuated vaccine. Another mechanism by which pre-existing antibodies

complexed with viral antigen may inhibit the activation of specific B cells is by cross-linking specific surface immunoglobulins and Fc-gamma receptors (Sarkar et al, 1996). But even in the absence of maternal antibodies, the immaturity of the immune system of infants early after birth precludes a high seroconversion rate (Gans *et al*, 2001), although the T cell reponse may be adequate (Siegrist, 2001).

In developing countries maternal antibodies seem to wane faster than thought until now. In a cross-sectional study from our own laboratory with Nigerian infants up to 9 months of age the overall prevalence of measles antibodies was 45 % with only 32% of the 3 months old and 2% of the 6-9 months old infants protected (Hartter *et al*, 2000). Other investigations also demonstrated an early loss of maternal measles antibodies in children from different developing countries with low national per-capita income. Seronegativity at 4 months of age was 52% in Brazzaville, Congo (Dabis *et al*, 1989), 54% in Kenya (Collaborative study by the Ministry of health of Kenya and WHO,1977), and 90% in Libreville, Gabon (Gendrel *et al*, 1986); at five months of age it was as high as 88% in Bangladesh (de Francisco *et al*, 1998) and 88% at six to seven months of age in South Africa (Kiepiela *et al*, 1991) and 93% in Ilorin, Nigeria (Babaniyi *et al*, 1995). In many of these studies, mothers were immune as a result of wild-type infection. In the first section of this study, we showed that vaccinees have considerably lower antibody titers than those protected by wild-type immunity (Damien *et al*, 1998). Therefore in the future, vaccinated mothers will transfer even less antibodies onto their babies. Moreover, some strains have been reported, that seem to be less susceptible to neutralization by vaccine induced antibodies (Klingle *et al*, 2000). Thus a number of factors contribute to a further reduction of child protection by maternal antibodies and to a widening of the "window of susceptibility" which delimits the time between the end of the protective immunity by maternal antibodies and the age at which the child is routinely vaccinated. This window of susceptibility is a period of high-risk for the child to develop complicated measles.

The development of new vaccines for vaccination of young infants during this period of enhanced susceptibility has been hampered by the concern for atypical measles, a severe form of measles with unusual clinical features that was observed after vaccination with the early killed vaccine (Brodsky, 1972). Although the exact mechanism leading to this disease is still unclear, several hypotheses have been produced. Initial studies of the immune response to killed measles vaccine, have shown that recipients developed antibodies to the H protein but little to the F or N proteins (Norrby *et al*, 1975; Norrby and Gollmar, 1975), indicating that the immune response against other proteins than H may be needed. With a better understanding of mechanisms of antigen presentation and T cell activation it seems more likely that an inadequate T cell response may be the cause of atypical measles. The cytotoxic class I restricted response is probably reduced after vaccination with killed vaccine. Moreover, an imbalance in cytokine production leading to a biased Th2 response is another suspected reason (Ward and Griffin, 1993). In monkeys, diseases mediated by immune complex formation and eosinophils in the presence of fusion-inhibiting antibody were observed (Polack et al, 1999). A similar model was also proposed to explain enhanced disease after vaccination with killed RSV virus (Openshaw *et al*, 2002). However, the

Th1/2 imbalance is not generally accepted, and some authors defend the possibility that the atypical measles could be caused by the absence or a marginal T cell reaction after immunization with a killed virus (Griffin D., personal communication).

While the latter hypothesis emphasizes the importance of strong T cell epitopes in an experimental vaccine, the Th1/Th2 imbalance suggests that no priming of MV-specific T cells may be the best approach. Our set of polyepitopes was constructed to take these views into account.

MV-specific antibodies are sufficient to protect against disease. For instance, hyperimmunoglobuline infusions protect until several days after infection (CDC, 1996; Gupta *et al*, 2001). Also infants are solely protected by maternal antibodies, highlighting the role of antibodies (Bromberg et al, 1994). The vaccine proposed here, is designed to induce antibodies against measles without priming for MV-specific T cells. The induction of an efficient immune response requires a T cell epitope. Therefore a broadly reactive promiscus T cell epitope of the tetanus toxoid, that activates T cells in the framework of most human MHC class II restriction elements was included in our sequence for priming in humans, along with a mouse ($H2^d$ restricted) T cell epitope demonstrated as very potent in our animal model (Brons *et al*, 1996), for experimental testing. It is very unlikely that such mouse short epitope sequence would also be a T cell epitope in many humans.

The B cell epitope is a mimotope, which does not share sequence homology with the measles protein, but mimicks a neutralising and protective conformational epitope of the fusion protein. One such mimotope has been described by Stewart *et al* (1995). The mimotope was selected with a monoclonal antibody directed against the fusion protein. The selection was performed using a library of randomly synthesized 8mer oligopeptides. The study from Stewart *et al* (1995) showed that the selected mimotope induced protective antibodies in the mouse model. The vaccine containing such mimotope would induce MV-specific neutralizing antibodies, but avoiding the induction of MV-specific T cell epitopes, thus preventing priming of the aberrant Th pathway that may be responsible for atypical measles.

As a result, polyepitopes based on the described epitopes should be able to induce the production of MV-specific antibodies without containing any MV-specific sequence, thus avoiding the induction of MV-specific T cells in human. Such setup would induce protective antibodies without being neutralized by remaining maternal antibodies and with limited risk of atypical measles.

The distance of antigenic regions, the importance of number, order and orientation of these epitopes have been discussed in the literature (Hathaway *et al*, 1995; El Kasmi *et al*, 1999). These factors play an important role in the efficiency of the immune response. In the presented approach, we have combined the mimotope and the T cell epitopes into a set of permutational polymimotope constructs with the intention to investigate the importance of the protein size, the

copy number and the relative position of the different subunits with respect to the antigenic and immunogenic properties of the recombinant protein. Adjacent or overlapping B and T cell epitopes have been observed in influenza hemagglutinin (Barnett *et al*, 1989), *Plasmodium falciparum* antigen Pf155/RESA (Troye-Blomberg *et al*, 1988) and other pathogens (Celada and Sercarz, 1988). In natural immunity, pairing of T and B cell epitopes uses a mechanism, which allows preferential processing and presentation of T cell epitopes adjacent to the antibody binding site. Through differential binding to epitopes of the same antigen, B cells could define the B cell epitope and can directly process (Davidson and Watts, 1989) and select the peptide fragment mediating T cell help as proposed by the concept of directional help (Celada and Sercarz, 1988). This requires an important role of B cells as antigen processing cells (APCs). B cells are directly involved as APCs, particularly when memory cells have already been established (Lanzavecchia, 1985).

A strategy similar to the one described here has already been developed by our laboratory by El Kasmi *et al* (2000) using peptides mimicking sequential MV-epitopes. El Kasmi *et al*. used the HNE and NE epitopes from the H protein to induce a neutralizing antibody response. These peptides actively induced virus-neutralizing antibodies even in the presence of protective levels of anti-MV antibodies. These same epitopes were also used to generate permutational polyepitopes (Theisen et al, 2000). At least one of these polyepitopes gave high neutralizing antibody titers (Bouche et al. submitted). Since in the latter studies peptides and polyepitopes were based on BCE derived from sequences of the MV, they potentially contain a T cell epitope active in a fraction of the human population. In contrast, the present approach completely avoids to include MV sequences as B cell epitopes.

Our constructs using the M2 epitope were produced in eukaryotic cells. T cell experiments showed that the constructs were able to stimulate T cells. This also indicated that the constructs were produced in the expression system. However, the mab did not detect any sizeable amounts of the construct in ELISA. Therefore, it was tested whether the Leu-Asp spacer (resulting from the restriction sites) between the mimotopes may have precluded recognition. An ELISA test with synthetic peptides and the monoclonal antibody F7-21 showed that the antibody recognized the Mimotope-Gly-Gly-Gly-Mimotope but did not detect the Mimotope-Leu-Asp-Mimotope peptide. This result suggested that the Leu-Asp spacer perturbs the secondary structure of the BCE in the synthetic peptide, preventing recognition by the antibody. The presence of the Leu-Asp amino acids flanking each subunit may explain the non-detection of the BCE in the recombinant protein and suggests that the spacer amino acids may also be responsible of a modified epitope conformation in the recombinant proteins. Numerous examples in the literature showed that the flanking regions of an epitope change its crossreactive-immunogenicity (Manca et al, 1993; Stittelaar *et al*, 2001; Hudecz, 2001) and it is very difficult to predict such changes.
Furthermore, the sera obtained with the Mimotope-Leu-Asp-Mimotope peptide did not show a significant titer of antibodies, indicating that the peptide was probably poorly immunogenic. Alternatively, it is possible that ionic interactions between the aspartic acid (Asp) situated in the

spacer and a positively charged amino acid (lysine or arginine) in the mimotope may have influenced its conformation in vivo, differently than in vitro. Cumulatively, these results suggest that the spacer used is not compatible with suitable crossreactive immunogenicity of the mimotope.

In studies by Steward and colleagues (Steward *et al,*1995; Obeid *et al*, 1995; Olszewska *et al*, 2000) the mimotope was described to induce antibodies that protected mice against an intracranial challenge with a rodent adapted measles virus. Therefore, this mimotope was considered as a possible candidate for a linear B cell epitope able to elicit neutralizing antibodies against the measles virus. However, when the F7-21 mab was tested for virus neutralising activity, it came to a surprise that the antibody did not neutralise the virus, casting some serious doubts on the protective capacity of both the antibody and the mimotope.
Steward *et al* (1995) obtained a neutralising mimotope from a non-neutralizing antibody. However, the literature shows that *in vivo* protection by antibodies, which do not neutralize *in vitro* is possible (McCullough *et al*, 1986; Gould *et al*, 1986; Harty *et al*, 1987; Burns *et al*, 1996). One notable example is the linear epitope of the G glycoprotein of the respiratory syncytial virus (RSV), described by Trudel *et al* (1991). However, this dichotomy has never been reported for the measles virus. Whether in the case of the F7-21 mab this could be explained by complement-mediated effector mechanisms or antibody-dependent cellular cytotoxicity (ADCC) and/or immune phagocytosis (Mochizuki *et al*, 1990) or the synergistic neutralising effect of several non-neutralising antibodies (Verrier *et al*, 2001) remains an open question.

Although a similar study from our laboratory with polyepitopes based on linear epitopes of MV gave some very promising results (El Kasmi *et al*, 2000; El Kasmi *et al*, 1999; Bouche et al, submitted), the present study showed that the mimotope M2 is not suitable for incorporation into such constructs possibly because of conformational incompatibility with the LD-spacer and possibly for other reasons too.

For futures projects, the current cloning strategie will still be used unchanged since we have not noticed a similar interference of the spacing amino acids with the desired conformation of other recombinant constructs in our laboratory. The effect impeding the recognition of mimotopes with Leu-Asp spacer by antibodies may therefore be related to the sequence particularities of the M2 mimotope only. Nonetheless these results illustrate that the neighboring amino acid environment can introduce important conformational modifications in the structure of a specific minimal epitope and the use of larger peptide fragments, presenting the core epitope in their natural sequence environment appears to be an interesting alternative to ensure the presentation of the desired epitope conformation in a recombinant protein.

IV.2.2 Polyepitopes derived from antigenic regions of the measles virus

As already discussed in the Chapter IV.1, the level of immunity of vaccinees is less robust than in individuals protected by wild-type immunity. This is certainly true when the comparison between vaccinees and late convalescents is based on antibody levels: antibody levels are both lower (Damien *et al*, 1998) and less durable in vaccinees (Mossong *et al*, 2000). At this point the significance of waning antibodies both for the protection of individuals and for the future epidemiology of measles virus remains unclear. While some authors are concerned by the loss of antibodies (Mossong *et al*, 2000) others expect that vaccinees with waning antibodies may still be protected by a more durable T cell immunity (Weidinger et al, 2000). For instance, it was observed that monkeys immunized with BCG expressing were substantially protected from lung inflammation by T cell immunity, in the absence of anti-MV antibodies (Zhu *et al*, 1997). Although T cells may prolong protections by vaccination beyond the prevalence of antibodies, the waning of immunity in vaccinees may develop into a major problem with respect to the WHO target of measles eradication. In the future, when the proportion of vaccinees with waning immunity is going to increase, there may be a need for revaccination of these individuals as well as those that are susceptible to secondary immune responses.

A strategy is proposed here for routine revaccination of vaccinees with waning immunity. In the absence of prior testing, antibody levels in these vaccinees are normally not known. Since these individuals may have variable levels of antibodies, the vaccine should also be efficient in the presence of antibodies. Ideally a vaccine designed for boosting vaccinees should be resistant to residual virus neutralising antibodies. Our vaccine strategy is based on the boosting of the T cell response by MV sequences of the main immunogenic viral proteins. Such a vaccine must include a large number of T cell epitopes and, at the same time, exclude functional neutralizing B cell epitopes. The vaccine is based on a polyepitope containing a large selection of known or putative CD4 and CD8 epitopes, many of them in their natural environments, facilitating the uptake and processing as described by Celada and Sercarz (1988). The large number of CD4+ T cell epitopes should ensure efficacy in the human population with polymorphic MHCs. Mice TCE were also included for ensuring testing in an animal model. Thus, selected sequences were assembled into single proteins, which have little or now resemblance with the 3D structure of the viral proteins. We expect that these chimeric proteins or polyepitopes would resist antibodies but boost the residual T cell response. The presence of numerous TCEs in the vaccine would also help to make the vaccine more resistant to immunological pressure and the generation of escape mutants.

Unlike the previous strategy, we used fragments from the N protein from measles. This protein carries a large number of CD4+ and CD8+ TCE (Nanan *et al*, 1995; Hickman *et al*, 1997; Beauverger *et al*, 1993; Fournier *et al*, 1996; Giraudon *et al*, 1988). Several studies suggested that recombinant proteins based on N protein epitopes alone may be protective although the N protein does not induce neutralizing antibodies. Bankamp *et al* (1991) have shown protection in Lewis rats against neurotropic MV, vaccinated with recombinant vaccinia virus expressing the nucleocapsid

protein. Immunized rats revealed polyclonal but not neutralizing antibodies to the N protein as well as an N protein-specific proliferative lymphocyte response. This study concluded that a CD4+ cell-mediated immune response specific for the N protein could be sufficient to control measles infections of the central nervous system (Bankamp et al, 1991). In this context, it is interesting to note that N protein has been reported to induce primarily antibodies of the IgG2a subclass after intraperitoneal injection, indicating a Th1 response (Cardoso *et al*, 1996; Olszewska *et al*, 2001). Similarly, other studies have shown that recombinant N protein was able to induce T cell responses and low levels of neutralizing antibody as well as considerably reducing the virus titer recovered from brain homogenates of mice (Fennelly *et al*, 1995).

Cytotoxic T-lymphocytes (CTL) against measles virus are thought to play an important role in recovery from disease (van Binnendijk *et al*, 1990). An and Whitton (1997) found that an isolated CTL epitope may confer protection against a lethal dose of a virus. In measles immunity, the major protein for CTL activity is the N protein and such CTL epitopes play an important role in the control of virus infection (Schadeck *et al*, 1999). However, other authors reported no reduction of virus titers in rats after immunization with N protein. (Schlereth *et al*, 2000b). These authors suggested that additional neutralizing epitopes may be required to overcome viral infection completely.

Some studies report that MV and CDV share an antigenically similar nucleoprotein which allows the MVN protein to protect dogs from distemper virus (Wild *et al*, 1993). Such vaccine containing numerous TCE from the N protein may also help to negate the selective pressure induced by current life-attenuated vaccine because of the high homology of the nucleoprotein between different strains or even different host species, preventing the emergence of resistant clades.

In the previous mimotope strategy, the spacers that resulted from the restriction sites were detrimental for antibody recognition, probably because of their influence on conformation. In the present strategy, virus-crossreactive antibodies were undesired. Therefore, the spacers may actually contribute by perturbing the conformation of the constructs. Since it is difficult to predict the effect of the spacer on processing, in particular, of class II-restricted TCEs, the same cloning strategies used for the construction of the polymimotopes was also utilized for the assembly of the polyepitope construct based on immunogenic sequences of the MV.

In this strategy, we have used the T7 prokaryotic expression system, hoping to increase the quantity of produced protein along with some experimental simplification. This expression system has been designed to produce high levels of recombinant proteins. It has been used in combination with a large variety of genes. However, sometimes proteins were produced in disappointingly small amounts, for reasons that were obvious in some cases and obscure in others (pET system manual, Novagen). By following the protocol recommended by the manufacturer, only low levels of recombinant protein were detected and considerable improvements were needed. In the case of the polyepitopes several reasons for the low expression must be considered: (I) low expression

efficiency of the T7 system for these randomly assembled proteins, or (ii) low stability and/or rapid degradation of the chimeric proteins, or (iii) a low sensitivity of the detection system.

IV.2.2.1 Optimization of the induction

The induction protocol provided by the manufacturer was recommended for generic proteins of smaller sizes (pET system manual, Novagen) than those of our constructs. Therefore, the protocol provided by the manufacturer may have been less optimal for larger constructs. Several attempts were made to improve the output of the induction process. The duration of induction was investigated and we showed that maximal levels of proteins were already obtained within 4 hours of induction. Induction times over 12 hours seemed to decrease the overal efficiency of the induction.

Different concentrations of IPTG were also experimented to find the best compromise between good induction and best cell growth. In addition, different experiments were made on the kinetics of delivery of IPTG. The best overall efficacy was obtained with a delivery of 1mM IPTG every 2 hours, during 4 hours. The fine-tuning of the purification method provided some amount of partially purified recombinant protein, sufficient to perform down-stream experiments.

Once the induction protocol was improved to produce the best overal performance, the obtained raw protein batch could be purified using a Nickel column connected on a HPLC.

IV.2.2.2 Protein stability

Some factors were described to influence the stability of the recombinant proteins such as the nature of the amino acid immediately following the N-terminal methionine (penultimate amino-acid).The amino-acid at this position determines the removal of the N-terminal fMet (formylmethionyl). This process is catalysed by a methionyl aminopeptidase and its rate decreases as the size of the penultimate amino acid side chain increases (Hirel *et al*, 1989; Lathrop *et al*, 1992). Little or no processing was observed by these authors when the following amino-acid occupied the penultimate position: His, Gln, Glu, Phe, Met, Lys, Tyr, Trp, Arg. Processing ranged from 16% to 97% when the other amino-acids occupied their critical position. In our cassette, the N terminal amino-acid sequence is the same for all constructs: The sequence, corresponding to the nucleotides running from the NcoI site (containing the ATG) to the Xba I site (first cloning site used during our cloning step) is Met - Ala - Ile - Leu – Ala, meaning that we have amino-acids with a relatively short side-chain, leading to a potentially high rate of removal of the fMet that may induce protein degradation.

Tobias *et al* (1991) have determined the relationship between a protein's amino-terminal amino acid and its stability in bacteria, i.e., the *N-end rule*. They reported protein half-lives of only 2 minutes when the following amino-acids were present at the amino-terminus: Arg, Lys, Phe, Leu, Trp and Tyr. In contrast, all other N-terminal amino acids conferred half-lives of >10 hours. As none

of these amino-acids are present in the immediate N-terminal end of our recombinant proteins, this suggests that our protein may have a relatively long half life.

The above studies suggest that for instance Leu in the penultimate position would be a poor choice, because it would likely be exposed by fMet processing and then be targeted for rapid degradation (Lathrop *et al*, 1992). Therefore, when an NdeI site is used for the production of unfused target proteins from pET vectors, Leu codons in the penultimate position should be avoided. Having always the same sequence at the beginning our our polyepitopes ensure the same treatment for all the proteins, along with a N-terminal region free of amino-acid inducing short half-life. Moreover, Leu codons in the penultimate position cannot be generated in our strategy because we used NcoI as the cloning site, where the penultimate codon must begin with a G.

Other factors that may also influence the expression level of target proteins include: mRNA secondary structure that may interfere with the ribosome binding site or the initiation codon (Tessier *et al*, 1984; Lee *et al*, 1987), or an excessive occurrence of rare codons in the target gene (Zhang *et al*, 1991; Sorensen *et al*, 1989), inducing the synthesis of truncated proteins. The codon usage of the constructs were compared for expression in human and bacteria and revealed no obvious incompatibilities.

IV.2.2.3 T Cell experiments

The T-cell experiments showed that the construct ABNP1-7 containing the epitope 185-199 of the nucleoprotein stimulated IL2 secretion of the corresponding T cell hybridomas. These experiments showed that at least one of the constructs was produced in detectable amounts (ABNP1-7). The lower stimulation induced by other constructs with the same NP fragment could be explained either by a lower expression efficiency possibly as a result of its larger size (ABNP1-7 is 444aa long and ABMVH1 is 729aa long) or by a perturbation by the MVH fragment that interfered with antigen processing.

IV.2.2.4 Antigenicity and immunogenicity studies

To detect the constructs produced in bacteria, monoclonal antibodies, which recognize both the native and the denatured H were used. Both antibodies are specific of the sequence 381-400 of the H protein; BH216 recognises the loop-shaped native form, whereas BH195 binds to the linear denatured form of the H protein. BH195 reacted with all constructs containing the related BCE, whereas the detection by BH216 was much weaker in the same constructs, indicating a poor conservation of the three-dimensional structure of this BCE (data not shown). Although, some of the proteins could be purified to some extend, human sera could not be used to test whether the constructs were recognized, because of a high background generated by contamination from residual bacterial components. Therefore, selected constructs were injected into mice and sera were tested for crossreactivity with the viral proteins. In ELISA, the constructs ABMVH1 and to a lesser extend ABNP1-7 induced sera that were able to bind denatured MV. When the same sera were tested against WMPT cells superinfected with MV, no binding above background levels was

observed. These cells are MV-infected and express native F and H proteins on the cell surface. The fact that the sera were positive in ELISA but not by flow cytometry, indicated that the epitopes recognized were not present on the cell surface-expressed MV proteins. This can be interpreted in either one of two ways: (I) the sera crossreacted with N protein, which is not detectable on the cell surface. It is very unlikely that the short distinct TCEs embedded in an unnatural environment mimic natural epitopes of the N protein. (ii) The sera react with denatured epitopes in the ELISA and do not recognize native epitopes of the MV on the cell surface of infected cells. This is the more likely interpretation of these results, although we can see that the AB MVH1 signal is much stronger than the AB NP1-7, indicating that the MVH1 fragment may play a direct or indirect role in the immune response.

ABMVH1 contained short sequences of the N protein, a relatively short central sequence of the F protein and a N- and –C terminally truncated domain of the H protein in the case of the construct. As expected these short sequence domains may not share conformational epitopes with the native full-length proteins, as was also indicated by the poor recognition of the BH216 antibodies. Alternatively, eukaryotic proteins produced in bacteria may undergo no or different post-translational modifications than those required for the immunogenicity of such proteins, including disulfide bonds formation, proteolytic processing of precursor proteins, failure to add chemical groups (e.g. glycosylation) or adding undesired groups (myristylation, palmitoylation, carboxylation, hydroxylation). Little is known about the effect of such groups on the protein structure.

These factors are of course more important in the case of conformation dependent B cell epitopes, as inadequate post-translational modifications may be less critical when they affect T cell epitopes. Although some glycopeptide antigens are presented by the MHC, the generation of peptide antigens from glycoproteins may require enzymatic removal of sugars before the protein can be cleaved (Rudd *et al*, 2001), therefore being less critical for T cell epitopes.

Some authors have already demonstrated the importance of the H protein sequence and structure for binding of monoclonal antibodies. It was shown that the absence of disulphide bonds impaired the capacity of the protein to react with mAbs specific for the discontinuous epitopes and thus its immunogenicity (Wilcox *et al*, 1988; Hu and Norrby, 1994). For the H protein, Hu *et al* (1994a) has suggested that two of the four individual carbohydrate side-chains have a large influence on the antigenicity of the molecule.Other studies have indicated that a large part of the maturation and processing of the H protein takes place in the ER (Hu *et al*, 1994b), suggesting that a part of the maturation process happening in the ER may not be reproducible in a bacterial environment.

Recent studies have also demonstrated that the uses of pET system with pelB leader sequence has led to the production of functionally and structurally conserved proteins, but the proteins needed further renaturation and oxidation (Ribo *et al*, 1996), thus generating more constraints to structurally sensible proteins, as the MV-H protein.

It has become clear that prokaryotes (Archaea and Bacteria) are capable of glycosylation of proteins (Moens and Vanderleyden, 1997), but only in a few cases is structural information available. Many different structures have been observed that display much more variation than that observed in eukaryotes. Due to their different cell structure, prokaryotes have to use glycosylation mechanisms different from those found in eukaryotes. Despite some similarities with the eukaryotic system, these differences may result in substantial differences in glycosylation patterns, with important consequences for the structure and immunogencity of proteins (Adeyefa *et al*, 1997; Jaskiewicz *et al*, 1994; Delmas and Laude, 1991; Caust *et al*, 1987).

Proof of principle of a strategy of vaccination in the presence of MV antibodies comes from our own laboratory, although in relation to another setting. El Kasmi et al. (2001) demonstrated that peptides that escape recognition by passively acquired anti-whole virus antibodies could potentially be used as components of a 'pre-vaccine' that could be given during early childhood irrespective of persisting maternal antibodies. Unlike vaccines based on recombinant proteins, such epitope-based peptide vaccines can be designed to be compatible with a subsequent boost with the standard life-attenuated vaccine. The same author demonstrates that MV wild-type isolates can be neutralized using synthetic peptides which are not recognized by neutralizing passive antibodies (El Kasmi *et al*, 2000; El Kasmi *et al*, 1999).

Experiments to test this in the presence of passively induced mouse antibodies against MV would not be conclusive: from other experiments in our laboratory, we know that MV preparations contain sufficient amount of denatured virus to induce antibodies against denaturated virus and therefore which cross-reacts with the constructs described here. Thus this can only be tested in animals in which the virus can replicate, such as the cotton rat or monkeys. Although, we did not have access to a monkey colony at the present time, collaborations to perform these experiments are now being seeked.

In summary, the chimeric constructs were designed to boost the measles specific T cell response in vaccinees with waning immunity. The above antigenicity and immunogenicity studies suggest that the constructs are most likely not recognized by anti-MV antibodies persisting in vaccinees. We have further demonstrated that our set of constructs was able to be adequately processed to generate the desired T cell epitopes and to stimulate T cells. This suggests that our T cell vaccine should be able to boost the T cell reponse in vaccinees without being neutralized by persisting antibodies, which would suppress the immune response to the vaccine.

Part V

Conclusion and Perspectives

V. Conclusions and perspectives

Before the introduction of a vaccine, 8 million people worldwide died each year from measles and there were an estimated 130 million cases. The use of the live, attenuated vaccine decreased the mortality by 88% within years. At present days, the WHO estimates that over 40 millions cases still occur worldwide each year, contributing to about 800.000 deaths, with the largest proportion of cases reported to occur in children under the age of 5 years.

Recovery from measles normally results in life-long protection (Panum, 1940) but, despite having reached high level of global vaccination against measles, outbreaks continue to occur in developing and developed countries (Frank *et al*, 1985; Gustafson *et al*, 1987), because the virus continues to circulate in seronegative individuals. However, there is also evidence that measles virus can circulate in seropositive populations (Pedersen *et al*, 1989; Gustafson *et al*, 1987; Ozanne and d'Halewyn, 1992; Pedersen *et al*, 1992). Secondary immune responses (SIR) against measles have been observed in fully immunized populations (Gustafson *et al*, 1987). It is reasonable to assume that people undergoing a clinically unapparent SIR after reexposure to measles are the most likely seropositive candidates to support transmission of the virus. It is therefore important to study the role of SIR-susceptibles for the epidemiology of measles.

In this study, we have characterized SIR in parents exposed to children with measles during an outbreak in 1996 (Muller *et al*, 1996a). Forty-five fully protected, measles late convalescent parents reexposed to measles, 4 (9%) developed an asymptomatic SIR with a significant increase in MV-specific IgG. SIR was only found in individuals with a pre-exposure IgG level below a well defined threshold and no individuals with immunity over that threshold seemed to be SIR susceptible. Moreover, such immunity increase was shown to be only transient, as the IgG levels of the 4 SIR individuals tends to return to original values 5 years after contact.

On the basis of the characteristics of SIR-susceptibles, we have estimated the frequency of SIR-susceptibles among individuals with natural and vaccine-induced immunity to define a high-risk population in which asymptomatic viral transmission could potentially be investigated. Such estimates may become important, since after vaccination antibody titers are lower and more likely to wane than after wild-type infection (Weibel *et al*, 1980; Krugman, 1983; Pedersen *et al*, 1986; Christenson and Böttiger, 1994).

Our study suggests that a subset of individuals which may be transiently contagious, can be serologically identified on the basis of their pre-existing immunity and that the estimation of their frequency could be relevant for future MV epidemiology and vaccination strategies. Other representatives populations have been investigated using the defined thresholds. Our study has demonstrated that vaccinated population had a much higher risk of being SIR susceptible (22.2-33.2% of the population) than individuals protected by natural immunity (3.2-3.9%).

Moreover, the vaccine-induced immunity of the older population wanes faster than natural immunity (Markowitz *et al*, 1990; Christenson and Böttiger, 1994), meaning that the proportion of SIR susceptible in a population where measles immunity is acquired only by vaccination could be potentially much higher that in our estimation. A study from Mossong *et al* (1999) demonstrated that after 25 years, 50% of seroconverted vaccinees would have an antibody titer below protective level. If the basic reproduction number of vaccinated SIR-susceptibles exceeds 1.24, elimination of measles would be made impossible using the current single-dose vaccination strategy (Mossong *et al*, 1999).

The role of SIR is therefore likely to grow in fully vaccinated populations where SIR could affect 30 % or more. Vaccination strategies as well as global eradication programs may have to consider the increasing number of SIR susceptibility in highly vaccinated populations, and this in addition to enhanced susceptibility due to waning immunity.

Therefore, the current research priorities of the WHO with respect to measles include, amongst others, the characterization of the immunobiology of the measles virus infection and immunization. This goal can be reached by studies of the protective immunity responses including the study of humoral and cell-mediated immunity in animals and man, the identification of the B and T cell epitopes of protective antigens and to investigate the immune response and characteristics of the infection during natural MV infection and immunization.

In our study, we have investigated two strategies aimed at two emerging problems of the current vaccination strategy. One is the window of susceptibility in young infants. More and more children became infected by measles before the age of vaccination. Earlier vaccination is complicated by the presence of maternal antibodies and there is a need for a vaccine that would be able to protect the seronegative children until vaccination age. The second strategy is aimed at vaccinees with waning immunity. With the increasing proportion of vaccinees in the population, the role of SIR is likely to grow. In this strategy, we proposed a set of designer proteins that should boost the T cell immunity without being neutralized by residual anti-MV antibodies.

The majority of infants are susceptible to measles by the age of 6-9 months in developing countries and 9-12 months in industrialized countries. Therefore, measles-related death is frequent (>50%) with children between 6 and 12 month (Aaby *et al, 1990).*

Current vaccination strategies recommend measles vaccination at 15 months of age in the developed countries and at 9 months in the developing world. The development of new vaccines for vaccination at earlier age is hampered by persisting transplacentally transferred maternal antibodies that neutralize the low dose of live viruses contained in the attenuated vaccine (Albrecht *et al*, 1977) and by the risks of atypical measles (Brodsky, 1972).

In this study, we demonstrated that vaccinees have considerably lower antibody titers than those protected by wild-type immunity (Damien *et al*, 1998). We can therefore suggest that vaccinated mothers will transfer even less antibodies onto their babies, leading to potentially higher measles-related mortality in infants below 9 months.

Such factors contribute to a further reduction of child protection by maternal antibodies and to a widening of the "window of susceptibility" which delimits the time between the end of the protective immunity by maternal antibodies and the age at which the child is routinely vaccinated.

Therefore, there is a need for a "pre-vaccine" that could be administered to young infants before the age of routine vaccination. The pre-vaccine proposed here was designed to be restistant to maternal antibodies, because it did not include any target sequence for measles neutralizing maternal antibodies. It was also supposed to be safe, with respect to atypical measles, because it did not include measles-specific human T cell epitopes. The B cell epitope of our construct was a mimotope, which does not share sequence homology with the measles protein, but mimicked a neutralizing and protective conformational epitope of the fusion protein.
The candidate vaccine was designed to induce MV-specific neutralizing antibodies, but avoiding the induction of MV-specific T cell epitopes. As a result, polyepitopes based on the described epitopes should be able to induce protective antibodies without being neutralized by residual antibodies and with limited risk of atypical measles.

Unfortunately, immunogenicity studies indicated that the spacer included in our polyepitopes induced conformational changes that prevented recognition by the monoclonal antibody used for the selection of the mimotope. Numerous examples in the literature showed that the flanking regions of an epitope could change its crossreactive immunogenicity (Manca et al, 1993; Stittelaar *et al*, 2001; Hudecz, 2001). Furthermore, the sera obtained with the immunization of mimotope peptide indicated that the peptide was not immunogenic, in contrast to the studies by Steward and colleagues (Steward *et al,* 1995; Obeid *et al*, 1995; Olszewska *et al*, 2000). In the studies from Steward *et al* (1995), the antibody used to select the mimotope was unable to neutralize the virus. Non-neutralizing antibodies can sometimes confer protection but this has not been reported for the measles virus. As the spacer used in our constructs rendered our constructs non-immunogenic and that the mimotope used was a questionable neutralizing epitope, it was decided to stop the study. This series of experiment served as a test bed for further series of experiments and demonstrated the importance of the flanking regions in the design of recombinant protein used as potential vaccines.

As demonstrated in our study, the level of immunity of vaccinees is less robust than in individuals protected by wild-type immunity. Antibody levels are both lower (Damien *et al*, 1998) and less durable in vaccinees (Mossong *et al*, 2000), although T cells may prolong protections by

vaccination beyond the prevalence of antibodies. The waning of immunity in vaccinees may become a major problem when the proportion of vaccinees in the population will increase, with respect to the WHO target of measles eradication.

Therefore, we also proposed a candidate vaccine designed for the revaccination of vaccinated individual with waning immunity as well as for individuals that are susceptible to secondary immune responses.

Ideally, a vaccine designed for boosting vaccinees should be resistant to residual virus neutralizing antibodies. Unlike our previous strategy, there is no risk of atypical measles in boosting the immunity of vaccinees, since they already have the correct T cell priming from their first vaccination. Our vaccine strategy is therefore based on the boosting of the T cell response by a large number of T cell epitopes of the main immunogenic viral proteins of MV by a protein that will not include functional neutralizing B cell epitopes. In this way, we expected that these chimeric proteins or polyepitopes would resist antibodies but boost the residual T cell response. The presence of numerous TCEs in the vaccine would also help to make the vaccine more resistant to immunological pressure and the generation of escape mutants by negating the selective pressure induced by current life-attenuated vaccine because of the high homology of the nucleoprotein between different strains or even different host species.

The selected constructs were injected into mice and sera were tested for crossreactivity with the viral proteins. Two constructs (ABMVH1 and to a lesser extend ABNP1-7) were shown to be able to induce MV cross-reactive sera. Such sera were unable to recognize H and F protein present on the surface of the WMPT cells superinfected with MV. This suggested that either the sera crossreacted with N protein in ELISA, which is not detectable on the cell surface of the WMPT proteins, or that the sera reacted with denatured epitopes in the ELISA and is in fact unable to recognize native epitopes of the MV.

Experiments to validate one of the two possibilities will require experiments in animal models were the virus can replicate. In such models, we would be able to test the eventual T cell proliferation generated by our construct and to investigate the presence of eventual anti-NP antibodies induced by our candidate vaccine.
The collected data suggests that the constructs are most likely not recognized by anti-MV neutralizing antibodies persisting in vaccinees. We have also shown T cell experiments where our constructs were adequately processed and stimulated specific T cell lines. This suggests that our T cell vaccine should be able to boost the T cell response in vaccinees without being neutralized by persisting antibodies, which would suppress the immune response to the vaccine.

With this series of studies, we have developed two possible strategies of design for candidates vaccines aimed at some of the limitation of the current vaccine strategy. These experimental

vaccines are trying to offer a long-term solution to the emerging problems of the current measles control campaign.

The future of vaccine development sits in the design of improved vaccines, designed using the techniques of molecular biology, based on viral or bacterial subunits. Novel vaccines should offer high sero-conversion rate and a long term protection along with low vaccine-related side-effects. Such vaccine should have a better heat-stability than life-attenuated measles vaccines, should be more resistant to maternal antibodies and could be more appropriate for revaccinating vaccinees that have lost protection as a result of waning immunity. Such vaccine should also have a long shelf-life, be easily administered, be eventually combined with vaccines of other diseases. They could explore alternative routes of immunization, for example oral administration, that would not require the involvement of trained health workers and avoid the risks of infection associated with needles injections.

The fulfillment of all those objectives will require many more years of research before usable vaccine candidates can be tested clinically. With this study, the authors hope to have made a small contribution towards this long term goal.

Part VI

References

VI. References

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Part VII

Abstracts

Design of an immunization strategy based on recombinant proteins of the measles virus in the context of a changing epidemiology

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Recovery from measles normally results in life-long protection, but despite having reached high level of global vaccination against measles, outbreaks continue to occur in developing and developed countries, because the virus continues to circulate in seronegative individuals. However, there is also evidence that measles virus can circulate in seropositive populations. Secondary immune responses (SIR) against measles have been observed in fully immunized populations and it is reasonable to assume that people undergoing a clinically unapparent SIR after reexposure to measles are the most likely seropositive candidates to support transmission of the virus.

Amongst 45 fully protected, measles late convalescent parents reexposed to measles, 4 (9%) developed an asymptomatic SIR with a significant increase in MV-specific IgG. SIR was only found in individuals with a pre-exposure IgG level below a well defined threshold. Moreover, such immunity increase was shown to be only transient. In combination with pre-exposure neutralizing and haemagglutination inhibiting titers, a threshold was defined below which SIR is likely to occur.

Our study suggests that a subset of individuals which may be transiently contagious, can be serologically identified on the basis of their pre-existing immunity and that the estimation of their frequency could be relevant for future MV epidemiology and vaccination strategies. Other representatives populations have been investigated using the defined thresholds. Our study shows that vaccinated population had a much higher risk of being SIR susceptible (22.2-33.2% of the population) than individuals protected by natural immunity (3.2-3.9%). Although viral transmission between protected individuals has never been directly demonstrated, the data describe a population in which protected but infections persons could potentially be of epidemiological importance.

In our study, we have investigated two strategies aimed at two emerging problems of the current vaccination strategy. One is the window of susceptibility in young infants. Vaccination before 9 months is made difficult by the presence of maternal antibodies and there is a need for a vaccine that would be able to protect the seronegative children until vaccination age, without risks of atypical measles. Our developed pre-vaccine was designed to be resistant to maternal antibodies, because it did not include any target sequence for measles neutralizing maternal antibodies. It was also supposed to be safe, with respect to atypical measles, because it did not include measles-specific human T cell epitopes. The B cell epitope of our construct was a mimotope, which does not share sequence homology with the measles protein, but mimicked a neutralizing and protective conformational epitope of the fusion protein. Unfortunately, immunogenicity studies indicated that the spacer included in our polyepitopes induced conformational changes that prevented recognition by the monoclonal antibody used for the selection of the mimotope. Furthermore, the sera obtained with the immunization of mimotope peptide indicated that the peptide was unable to induce neutralizing antibodies.

In a second part of our study, we proposed a candidate vaccine designed for the revaccination of vaccinated individual with waning immunity as well as for individuals that are susceptible to secondary immune responses. Our vaccine strategy was therefore based on the boosting of the T cell response by a large number of T cell epitopes of the main immunogenic viral proteins of MV by a protein that will not include functional neutralizing B cell epitopes, to prevent neutralization by remaining antibodies. Two constructs were shown to be able to induce MV cross-reactive but not neutralizing sera. We have also shown T cell experiments where our constructs were adequately processed and stimulated specific T cell lines. This suggests that our T cell vaccine should be able to boost the T cell response in vaccinees without being neutralized by persisting antibodies, which would suppress the immune response to the vaccine.

Mise au point d'une stratégie d'immunisation basée sur des protéines recombinants du virus de la rougeole, dans le contexte d'une épidémiologie changeante

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La guérison de la rougeole résulte normalement en une protection à vie, mais, malgré avoir atteint de hauts niveaux de vaccination contre la rougeole dans le monde, des épidémies continuent à apparaître aussi bien dans les pays en voie de développement que dans les pays développés, principalement parce que le virus continue de circuler grâce aux individus séronégatifs. Néanmoins, il y a également des preuves que le virus peut circuler dans le sang d'individus séropositifs. Des réponses immunitaires secondaires (RIS) contre la rougeole ont déjà été observées dans des populations totalement immunisées et on peut penser que des individus qui subissent une réponse immunitaire secondaire asymptomatique après réexposition à la rougeole sont les plus sérieux candidats séropositifs pouvant être responsables de la transmission du virus.

Parmi 45 parents protégés, ayant contracté la rougeole dans leur jeunesse, 4 (9%) ont développé une RIS avec une augmentation significative de leurs IgG spécifiques à la rougeole. Cette RIS a été détectée uniquement chez des individus avec un niveau d'IgG avant exposition inférieur à une limite bien définie. De plus, cette croissance du niveau de leur immunité était seulement temporaire. En combinant IgG, titres de neutralisation et d'inhibition d'hémagglutination, un niveau a été défini, en dessous duquel une RIS était susceptible de se produire.

Notre étude suggère qu'un groupe d'individus, qui peut être temporairement contagieux, peut être défini sérologiquement sur base de leur niveau d'immunité à la rougeole et que l'estimation de leur susceptibilité à la RIS pourrait être importante pour de futures études épidémiologiques de la rougeole et pour l'élaboration de stratégies de vaccination. D'autres populations représentatives ont été étudiées en utilisant les limites définies précédemment. Notre étude montre qu'une population vaccinée a un risque plus élevé de susceptibilité à la RIS (22.2-33.2% de la population) que des individus qui ont acquis leur immunité naturellement (3.2-3.9%). Bien que la transmission virale entre individus immunisés n'ait jamais été prouvée, nos données décrivent une population dans laquelle des individus protégés mais potentiellement infectieux pourraient avoir une importance du point de vue épidémiologique.

Dans la suite de notre étude, nous avons expérimenté deux stratégies visant deux problèmes émergents des stratégies de vaccinations actuelles. L'un est la fenêtre de susceptibilité chez les jeunes enfants. La vaccination avant l'âge de 9 mois est rendue difficile par la présence d'anticorps maternels, et il y a donc un besoin pour un vaccin capable de protéger ces enfants séronégatifs jusqu'à l'âge de vaccination, sans risques de provoquer une rougeole atypique. Le pré-vaccin que nous avons développé a été étudié pour être résistant aux anticorps maternels car il n'inclut pas d'épitopes étant la cible d'anticorps neutralisants. Il est également supposé être sûr, du point de vue de la rougeole atypique, car il ne comporte pas d'épitopes T humains spécifiques de la rougeole. L'épitope B utilisé dans nos épitopes était un mimotope, qui n'a pas de séquence commune avec des protéines du virus de la rougeole, mais qui imite la conformation tridimensionnelle d'un épitope conformationnel décrit comme neutralisant et protecteur. Malheureusement, nos études immunologiques nous ont indiqué que l'élément espaceur utilise dans nos constructions provoquait des changements de conformation et empêchait sa reconnaissance par notre anticorps monoclonal utilisé pour la sélection du mimotope. De plus, le sérum obtenu par l'immunisation du mimotope nous a permis de démontrer que ce peptide était incapable d'induire la production d'anticorps neutralisants.

Dans une seconde partie de notre étude, nous avons proposé un candidat vaccin destiné à la revaccination d'individus vaccinés et ayant perdu leur immunité, ainsi que pour des individus susceptibles de faire une RIS. Notre stratégie de vaccination était donc basée sur la restimulation de leur réponse lymphocytaire T par une protéine recombinante contenant un grand nombre d'épitopes T provenant des protéines de rougeole les plus immunogènes, mais pas d'épitope B fonctionnel. Deux protéines recombinantes ont montré qu'elles étaient capables d'induire des anticorps qui réagissaient également avec la rougeole mais sans être neutralisants. Nous avons également montré dans une expérience de prolifération de lymphocytes T que nos protéines recombinantes étaient correctement traitées et stimulaient des lignées de lymphocytes T spécifiques. Cela suggère que notre vaccin à lymphocyte T devrait être capable de restimuler la réponse lymphocytaire T des vaccinés mais sans être neutralisé par les anticorps restants, ce qui éliminerait toute réponse possible contre le vaccin.